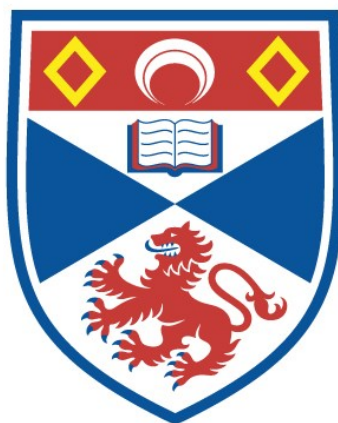


THE VERTEBRATE NEURONAL GAMMA-  
AMINOBUTYRIC ACID (GABAA) RECEPTOR AND ITS  
MODULATION: A PATCH CLAMP STUDY

Dineshkumar Mistry

A Thesis Submitted for the Degree of PhD  
at the  
University of St Andrews



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THE VERTEBRATE NEURONAL  $\gamma$ -AMINOBUTYRIC ACID (GABA<sub>A</sub>)  
RECEPTOR AND ITS MODULATION: A PATCH CLAMP STUDY.

BY: DINESHKUMAR MISTRY



Th A734

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#### ACKNOWLEDGMENTS

I would like to thank my supervisor, Prof. G.A. Cottrell for his help and advice throughout this project. I thank Mr Brian Powell for help with culturing of the neurones and various other people in the department who are too numerous to mention but especially those in the animal house. I would like to thank my colleagues in pharmacology for their encouragement. I acknowledge the financial support of the MRC, the DHSS and the Russell Trust for financial help in attending a conference.

Finally, I would like to thank my family for their encouragement during this study. Special mention is made to my parents and sister Surekha for financial help during my BSc degree at the University of East Anglia, Norwich which enabled me to undertake this study. I would like to dedicate this thesis to my family.

## ABSTRACT

Pressure application of  $\gamma$ -aminobutyric acid (GABA) to mouse spinal and rat DRG neurones maintained in culture evoked transient membrane currents. Using the whole-cell patch clamp technique, these currents were shown to primarily involve the flow of  $\text{Cl}^-$ . The GABA-evoked whole-cell currents in both types of neurones were reversibly suppressed by the GABA<sub>A</sub> antagonist bicuculline. The barbiturate phenobarbitone reversibly potentiated GABA-evoked whole-cell currents in mouse spinal neurones.

Attempts to look at the unitary currents activated by GABA in outside-out patches, revealed spontaneous unitary currents. The I-V relationships of the spontaneous currents were linear and had a reversal potential of 0mV in symmetrically distributed  $\text{Cl}^-$  solutions. Changing the monovalent cation concentrations on one or both sides of the membrane patch had no effect on the amplitude or the reversal potential of the spontaneous currents. Replacing some of the  $\text{Cl}^-$  in the patch pipette with the impermeant anion  $\text{SO}_4^{2-}$  shifted the reversal potential to more negative values. These spontaneous currents in both types of neurones were blocked by bath perfusion of bicuculline. GABA-activated unitary currents in outside-out patches, the main conductance state in both types of neurones was 30pS. However, GABA could occasionally also activate other conductance levels. Spontaneous  $\text{Cl}^-$  currents did not occur in cell-attached patches from mouse spinal and rat DRG neurones, suggesting that the spontaneous events in the outside-out patches did not represent the activity of voltage dependent  $\text{Cl}^-$  channels.

Alphaxalone, a steroid anaesthetic, potentiated GABA-evoked whole cell currents in both spinal and DRG neurones. At high ( $\mu\text{M}$ ) concentrations, pressure application of alphaxalone evoked a membrane  $\text{Cl}^-$  current; this current was reversibly suppressed by bicuculline and potentiated by phenobarbitone. Pregnanolone (5 $\beta$ -pregnane-3 $\alpha$ -ol-20-one) a progesterone metabolite at low (nM) concentrations reversibly enhanced GABA currents in spinal neurones. Pregnanolone at higher concentrations pressure applied to spinal neurones had a weak direct agonist action on the GABA<sub>A</sub> receptor. Pregnanolone prolonged the burst duration of GABA-activated unitary currents in outside-out patches from spinal neurones.

Some of the actions of the steroids on the GABA<sub>A</sub> receptor were very similar to the barbiturates, bemegride, a respiratory stimulant was formerly used clinically to counteract barbiturate poisoning in man. Experiments were conducted to see whether bemegride could be used as a specific barbiturate antagonist. Bemegride reduced phenobarbitone enhanced GABA currents in mouse spinal neurones. However, bemegride alone also reduced GABA and pentobarbitone evoked currents to a similar extent. This is suggestive of a noncompetitive action on the GABA<sub>A</sub> receptor, therefore it was not used to elucidate the site of action of steroids.



# ABBREVIATIONS

$a_{Cl}$	chloride activity
approx.	approximately
$B_{max}$	density of binding sites
BZ	benzodiazepine
$\beta$ -CCE	beta carboline-3-carboxylate ethyl ester
$\beta$ -CCM	beta carboline-3-carboxylate methyl ester
$\beta$ -CCPr	beta carboline-3-carboxylate propyl ester
$Ca^{2+}$	calcium ion
Cl	chloride
$Cl^{-}$	chloride ions
$[^{36}Cl^{-}]$	radioactively labelled chloride ion
$CO_2$	carbon dioxide
CNS	central nervous system
DBI	diazepam binding inhibitor
DMCM	methyl-6, 7, dimethoxy 4 ethyl- $\beta$ -carboline-3-carboxylate
DRG	Dorsal Root Ganglion
$E_{Cl}$	chloride equilibrium potential
$E_K$	potassium equilibrium potential
EGTA	ethylene glycol bis( $\beta$ -aminoethylether) N,N',N''N'teracetic acid
FuDR	fluoro-2-deoxyuridine.
GABA	gamma-aminobutyric acid
GHK	Goldmann-Hodgkin-Katz
$\gamma$	single channel conductance
HEPES	N-2-Hydroxyethylpiperazine N'-2-ethanesulphonic acid
HPLC	high performance liquid chromatography
I	current
ipsc	inhibitory postsynaptic current
ipsp	inhibitory postsynaptic potential
$K_d$	dissociation constant

$K^+$	potassium ion
K	potassium
$Na^+$	sodium ion
NGF	nerve growth factor
PBS	phosphate buffered saline
pregnanolone	5 $\beta$ -pregnane-3 $\alpha$ -ol-20-one
pregnanedione	5 $\beta$ -pregnane-3, 20-dione
3 $\alpha$ -OHDHP	3 $\alpha$ -hydroxy-5 $\alpha$ -dihydroprogesterone
3 $\alpha$ -THDOC	3 $\alpha$ 5 $\alpha$ tetrahydrodeoxycorticosterone
Ro5-4864	5(4-chlorophenyl) diazepam
Ro15-1788	ethyl-8-fluoro5,6 dihydro5methyl-6-oxo-(4H)-imidazo [1,5a] [1,4a] benzodiazepine-3-carboxylate.
V	potential
$V_h$	holding potential
$V_m$	membrane potential
$V_p$	patch potential
$V_{\text{pipette}}$	pipette potential

#### UNITS

G $\Omega$	gigaohm
M $\Omega$	megaohm
M	molar
mM	millimolar
nM	nanomolar
$\mu$ M	micromolar
s	second
ms	millisecond
V	volt
mV	millivolt

m	metre
µm	micrometre
mm	millimetre
cm	centimetre
pA	picoampere
nA	nanoampere
ml	millilitre
µg	microgramme
pS	picosiemens

## SUMMARY

1. Using the whole-cell patch clamp technique, pressure application of GABA (10-100 $\mu$ M) evoked transient membrane currents in mouse spinal and rat DRG neurones maintained in culture.

2. These whole-cell currents in both types of neurones were shown to involve the flow of  $\text{Cl}^-$ , by replacing some of the  $\text{Cl}^-$  in the patch pipette with the impermeant anion  $\text{SO}_4^{2-}$ . The reversal potential of the currents shifted to a value close to the calculated  $E_{\text{Cl}}$ .

3. The GABA-evoked whole-cell currents were reversibly suppressed by the GABA<sub>A</sub> antagonist bicuculline, suggesting that the GABA response was of the GABA<sub>A</sub> type.

4. The barbiturate phenobarbitone reversibly enhanced GABA-evoked currents in mouse spinal neurones.

5. The benzodiazepine agonist diazepam also reversibly enhanced GABA-evoked whole-cell currents in mouse spinal neurones.

6. Outside-out patches isolated from the somato of mouse spinal and rat DRG neurones exhibited spontaneous unitary currents.

7. These unitary currents had various different conductances ranging from 6-100pS in mouse spinal neurones and 6-67pS in rat DRG neurones.

8. Replacing the main monovalent cations on one or both sides of the membrane patch with the impermeant cations Tris/choline did not affect the amplitude or the reversal potential of the spontaneous currents.

9. Replacing some of the  $\text{Cl}^-$  in the patch pipette with the impermeant anion  $\text{SO}_4^{2-}$  shifted the reversal potential close to the calculated  $E_{\text{Cl}}$ .

10. Exposure of the outside-out patches to  $10\mu\text{M}$  bicuculline blocked the spontaneous current activity.

11. No spontaneous  $\text{Cl}^-$  currents were obtained in cell-attached patches from mouse-spinal and rat DRG neurones.

12. GABA-activated unitary currents were recorded in cell-attached patches from rat DRG neurones.

13. Alphaxalone, a steroid anaesthetic, reversibly potentiated GABA-evoked whole-cell currents in mouse spinal and rat DRG neurones.

14. At higher ( $>1\mu\text{M}$ ) concentrations, alphaxalone directly evoked a membrane  $\text{Cl}^-$  current in spinal neurones; this current was reversibly suppressed by bicuculline and enhanced by phenobarbitone.

15. Pregnanolone ( $5\beta$ -pregnane- $3\alpha$ -ol-20-one) a progesterone metabolite, at low (nM) concentrations reversibly enhanced GABA currents in spinal neurones.

16. At higher concentrations pregnanolone had a weak direct agonist action on the GABA<sub>A</sub> receptor.

17. Pregnanolone (200/300nM) prolonged the burst duration of GABA-activated unitary currents in outside-out patches from spinal neurones.

18. Some of the actions of the steroids were very similar to those of barbiturates, bemegride, a respiratory stimulant was formerly used to counteract barbiturate poisoning in man.

19. Experiments to determine whether bemegride could be used as a barbiturate antagonist were performed.

20. Bemegride reduced phenobarbitone enhanced GABA currents in spinal neurones.

21. Bemegride, alone, antagonized GABA and pentobarbitone evoked whole-cell currents.

22. Bemegride appeared to have a noncompetitive action on the GABA<sub>A</sub> receptor.

## CONTENTS

### CHAPTER 1 INTRODUCTION

1.1	SYNAPTIC TRANSMISSION	1
1.2	GABA AS AN INHIBITORY TRANSMITTER	2
1.3	DISTRIBUTION OF GABA <sub>A</sub> RECEPTORS IN THE CENTRAL NERVOUS SYSTEM	5
1.4	INHIBITORY ACTION OF GABA IN VERTEBRATES	6
1.5	GABA <sub>B</sub> RECEPTORS	8
1.6	GABA <sub>A</sub> RECEPTORS	11
1.7	BENZODIAZEPINE RECEPTORS	12
1.7.1	PERIPHERAL TYPE BENZODIAZEPINE RECEPTOR	13
1.7.2	CENTRAL TYPE BENZODIAZEPINE RECEPTOR	15
1.7.3	POSSIBLE SUBTYPES OF THE CENTRAL BENZODIAZEPINE RECEPTOR	16
1.7.4	ACTIONS OF BENZODIAZEPINES ON MEMBRANE CURRENTS	17
1.7.5	MOLECULAR MECHANISM OF ACTION OF BENZODIAZEPINES	22
1.7.6	ENDOGENOUS BENZODIAZEPINE LIGANDS	24
1.8	BARBITURATES	26
1.8.1	RADIOLIGAND BINDING STUDIES OF BARBITURATES	27
1.8.2	ACTIONS OF BARBITURATES ON MEMBRANE CURRENTS	29
1.8.3	MOLECULAR MECHANISM OF ACTION OF BARBITURATES	32
1.9	INVERTEBRATE GABA RECEPTORS	33
1.9.1	MODULATION OF GABA RESPONSES IN INVERTEBRATES	36
1.10	STEROID MODULATION OF THE MAMMALIAN GABA <sub>A</sub> RECEPTOR	39
1.10.1	OTHER ACTIONS OF STEROIDS ON MEMBRANE CURRENTS	43
1.10.2	STEREOSELECTIVITY OF STEROID ACTION	44
1.10.3	MECHANISM OF ACTION OF THE STEROIDS	46
1.10.4	PHYSIOLOGICAL SIGNIFICANCE OF STEROID ACTION	48
1.11	GABA ACTIVATED SINGLE CHANNEL CURRENTS IN VERTEBRATES	50
1.12	OTHER AGENTS INTERACTING WITH THE GABA <sub>A</sub> RECEPTOR	51
1.13	SUITABILITY OF DISSOCIATED NEURONES MAINTAINED IN CULTURE	54
1.14	AIMS AND OBJECTIVES	56

## CHAPTER 2 METHODS

2.1	PREPARATION AND DISSOCIATION OF MOUSE SPINAL NEURONES	57
2.2	COLLAGEN COATING OF PLATES	60
2.3	MAINTENANCE OF CULTURES	60
2.4	DEVELOPMENT AND MORPHOLOGY OF NEURONES	61
2.5	PREPARATION OF RAT DRG NEURONES	62
2.6	PATCH CLAMP METHODS	64
2.7	GLASS CAPILLARIES FOR PIPETTES	65
2.8	PULLING PIPETTES	66
2.9	COATING PIPETTES WITH SYLGARD	67
2.10	HEAT POLISHING PIPETTES	67
2.11	BUBBLE TEST	68
2.12	FILLING PATCH PIPETTES	68
2.13	PATCH CLAMP APPARATUS	69
2.14	ARRANGEMENT OF APPARATUS FOR PATCH-CLAMP RECORDING	70
2.15	CELL-ATTACHED PATCH FORMATION	71
2.16	WHOLE-CELL RECORDING	72
2.17	OUTSIDE-OUT PATCH FORMATION	72
2.18	DATA COLLECTION AND ANALYSIS	73
2.19	PRESSURE APPLICATION OF DRUGS	74
2.20	PERFUSION SYSTEM	76
2.21	SOLUTIONS	76
2.22	LIQUID JUNCTION POTENTIALS	78
2.23	DRUGS	79

## CHAPTER 3 RESULTS

3.1	VOLTAGE RECORDINGS OF MOUSE SPINAL AND RAT DRG NEURONES	80
3.1.1	MOUSE SPINAL NEURONES	80
3.1.2	RAT DRG NEURONES	81
3.2	BASIC PHARMACOLOGY OF GABA-EVOKED WHOLE-CELL CURRENTS	82
3.3	IONIC MECHANISM OF THE GABA RESPONSE (WHOLE-CELL STUDIES)	84
3.3.1	MOUSE SPINAL NEURONES	84



3.3.2	RAT DRG NEURONES	86
3.4	SPONTANEOUS UNITARY CURRENTS FROM MOUSE SPINAL NEURONES	87
3.4.1	SPONTANEOUS UNITARY CURRENTS IN SYMMETRICAL $\text{Cl}^-$ SOLUTIONS	87
3.4.2	LACK OF EFFECT OF CATIONS ON SPONTANEOUS UNITARY CURRENTS	89
3.4.3	SPONTANEOUS UNITARY CURRENTS IN ASYMMETRICAL $\text{Cl}^-$ SOLUTIONS	92
3.4.4	TRANSITIONS BETWEEN DIFFERENT CONDUCTANCE STATES	93
3.4.5	EFFECT OF BICUCULLINE ON SPONTANEOUS UNITARY CURRENTS	94
3.5	GABA-ACTIVATED UNITARY CURRENTS IN MOUSE SPINAL NEURONES	94
3.5.1	MULTIPLE CONDUCTANCE STATES ACTIVATED BY GABA IN EXCISED PATCHES	95
3.6	SPONTANEOUS UNITARY CURRENTS FROM RAT DRG NEURONES	96
3.6.1	SPONTANEOUS UNITARY CURRENTS IN SYMMETRICAL $\text{Cl}^-$ SOLUTIONS	96
3.6.2	LACK OF EFFECT OF CATIONS ON SPONTANEOUS UNITARY CURRENTS	97
3.6.3	TRANSITIONS BETWEEN DIFFERENT CONDUCTANCE STATES	98
3.6.4	SPONTANEOUS UNITARY CURRENTS IN ASYMMETRICAL $\text{Cl}^-$ SOLUTIONS	99
3.6.5	EFFECT OF BICUCULLINE ON SPONTANEOUS UNITARY CURRENTS	99
3.7	GABA-ACTIVATED UNITARY CURRENTS IN RAT DRG NEURONES	100
3.8	EFFECT OF DIAZEPAM ON GABA-EVOKED WHOLE-CELL CURRENTS	100
3.9	STEROID ACTION ON THE GABA <sub>A</sub> RECEPTOR (WHOLE-CELL STUDIES)	102
3.9.1	POTENTIATION OF THE GABA CURRENT BY ALPHAXALONE	102
3.9.2	DIRECT AGONIST ACTION OF ALPHAXALONE ON THE GABA <sub>A</sub> RECEPTOR	103
3.9.3	EFFECT OF BICUCULLINE AND PHENOBARBITONE ON ALPHAXALONE CURRENT	104
3.10	EFFECT OF ENDOGENOUS STEROIDS	105
3.10.1	POTENTIATION OF GABA-EVOKED CURRENTS BY PREGNANOLONE	105
3.10.2	WEAK DIRECT AGONIST ACTION OF PREGNANOLONE	106
3.11	UNITARY CURRENT STUDIES	106
3.11.1	MECHANISM OF ACTION OF PREGNANOLONE POTENTIATION	106

3.11.2	INABILITY TO OBSERVE STEROID-EVOKED UNITARY CURRENTS	108
3.12	EFFECT OF BETAXALONE (A $\beta$ -HYDROXY ISOMER OF ALPHAXALONE)	109
3.12.1	LACK OF EFFECT OF BETAXALONE ON GABA WHOLE-CELL CURRENTS	109
3.12.2	LACK OF DIRECT AGONIST ACTION BY BETAXALONE	110
3.13	EFFECT OF BEMEGRIDE ON GABA <sub>A</sub> RECEPTOR (WHOLE-CELL STUDY)	110
3.13.1	ACTION OF BEMEGRIDE ON PHENOBARBITONE ENHANCED GABA CURRENTS	111
3.13.2	REDUCTION OF GABA CURRENTS BY BEMEGRIDE	111
3.13.3	PENTOBARBITONE-EVOKED WHOLE-CELL CURRENTS	112
3.13.4	REDUCTION OF PENTOBARBITONE CURRENTS BY BEMEGRIDE	112
3.14	CELL-ATTACHED PATCH EXPERIMENTS	113
3.14.1	CELL-ATTACHED PATCHES FROM MOUSE SPINAL NEURONES	114
3.14.2	CELL-ATTACHED PATCHES FROM RAT DRG NEURONES	115
3.14.3	EFFECT OF HIGH (300mM) K <sup>+</sup> IN THE PATCH PIPETTE	116
3.14.4	GABA-ACTIVATED UNITARY CURRENTS IN CELL-ATTACHED PATCHES	117
CHAPTER 4 DISCUSSION		
4.1	IONIC NATURE OF THE GABA-EVOKED WHOLE-CELL CURRENTS	120
4.2	SPONTANEOUS CURRENTS IN EXCISED PATCHES FROM MOUSE SPINAL NEURONES	122
4.3	SPONTANEOUS CURRENTS IN EXCISED PATCHES FROM DRG NEURONES	126
4.4	CELL-ATTACHED PATCH EXPERIMENTS	127
4.5	GABA-ACTIVATED UNITARY CURRENTS IN MOUSE SPINAL NEURONES	130
4.6	GABA-ACTIVATED UNITARY CURRENTS IN RAT DRG NEURONES	131
4.7	SPONTANEOUS UNITARY CURRENTS OBSERVED BY OTHER WORKERS	132
4.8	SPONTANEOUS LARGE CONDUCTANCE LEVELS	134
4.9	SIMILARITIES BETWEEN SPONTANEOUS EVENTS AND GABA-ACTIVATED CURRENTS	136
4.10	POSSIBLE EXPLANATIONS FOR THE SPONTANEOUS EVENTS	138
4.11	EFFECT OF DIAZEPAM ON GABA-EVOKED WHOLE-CELL CURRENTS	142
4.12	EFFECT OF STEROIDS ON THE GABA <sub>A</sub> RECEPTOR	144
4.13	MECHANISM OF POTENTIATION OF GABA RESPONSES BY STEROIDS	146

4.14	WEAK DIRECT AGONIST ACTION OF ENDOGENOUS STEROIDS	149
4.15	SITE OF ACTION OF STEROIDS	150
4.16	ACTION OF BEMEGRIDE ON THE GABA <sub>A</sub> RECEPTOR	152
	REFERENCES	156
	APPENDIX	199



## INTRODUCTION

## INTRODUCTION

### 1.1 SYNAPTIC TRANSMISSION

Synaptic transmission occurs through two different mechanisms, one of which is electrical and the other chemical. At electrical synapses, currents generated in the presynaptic nerve terminal spread directly to the next neurone through a low resistance pathway. This low resistance pathway is provided by gap junctions, these are regions in which the two membranes are brought into close apposition (2-3nm). A direct connection between these two membranes is provided by protein molecules located in the bilayers, these protein molecules allow current to pass from one cell to another.

In chemical synapses, the presynaptic and postsynaptic membranes are not directly connected, and transmission occurs via the release of specific compound(s), the neurotransmitter(s) from the presynaptic terminal. Normally the compound diffuses quickly across the synaptic gap to the postsynaptic cell, where it changes the permeability of the postsynaptic membrane to specific ions producing either an excitatory or inhibitory synaptic potential. The type of ion(s) carrying the synaptic current and their equilibrium potentials relative to the threshold for action potential initiation determines whether the responses are excitatory or inhibitory. An example of an excitatory response occurs at the vertebrate neuromuscular junction (Anderson and Stevens, 1973). Here, the chemical transmitter acetylcholine produces a simultaneous increase in the permeability of the postsynaptic



membrane to sodium and potassium ions leading to a depolarizing postsynaptic potential. The permeability to chloride or potassium ions is generally increased at inhibitory synapses, this increase shifts the membrane potential away from threshold.

Neurons in the central nervous system receive both excitatory and inhibitory inputs; the interaction of these two components determines whether a nerve impulse is generated. As well as such relatively fast synaptic potentials, slow synaptic potentials are also observed in the central nervous system and can also be either excitatory or inhibitory. Usually these slow potentials have a delayed onset, and can last for several seconds or minutes and may involve either an increase or decrease in membrane permeability. An example of this occurs in mammalian sympathetic ganglion cells where acetylcholine produces a slow excitatory potential lasting for 30-60 seconds, this response is thought to involve a decrease in  $K^+$  membrane permeability (Kuffler, 1980). These changes in permeability often involve intracellular mediated effects (for review see, Kuffler and Nicholls, 1984).

## 1.2 GABA AS AN INHIBITORY NEUROTRANSMITTER

$\gamma$ -aminobutyric acid (GABA) was first identified in the vertebrate nervous system by Roberts and Frankel (1950) using paper chromatography. The role of GABA remained unknown for several years. An inhibitory neurotransmitter function for GABA was proposed from work with peripheral invertebrate systems. GABA was found to be the major factor in brain extracts responsible for the inhibitory action

of these extracts on the crayfish stretch receptor system (Bazemore, Elliot and Florey, 1957).

GABA at low concentrations had effects on the crayfish stretch receptor system that were remarkably similar to those found on stimulation of the inhibitory nerve (Kuffler and Edwards, 1958). These findings suggested that GABA might be the inhibitory transmitter and supporting evidence was obtained when the inhibitory axons were found to contain large concentrations of GABA (0.1M), while less than 1% of these levels could be detected in excitatory nerves (Kravitz, Kuffler and Potter, 1963).

GABA was also released from inhibitory nerves innervating the opener muscle of the lobster claw, in amounts proportional to the number and frequency of stimuli applied to the nerves (Otsuka, Iversen, Hall and Kravitz, 1966). Glutamic acid decarboxylase (GAD), the enzyme responsible for GABA synthesis was also preferentially distributed in inhibitory nerves (Hall, Bownds, and Kravitz, 1970). Picrotoxin, a convulsant agent, blocked the inhibitory action of GABA and the natural inhibitory transmitter similarly (Van der Kloot and Robbins, 1959).

The work of Boiestal and Fatt (1958) and Takeuchi and Takeuchi (1967) demonstrated that the inhibition of the muscular contraction was caused by a selective increase in the muscle membrane conductance to chloride ions. Both applied GABA and nerve stimulation shifted the postsynaptic membrane potential towards  $E_{Cl}$ , the chloride equilibrium potential. Taken together, these reports met all the requirements for GABA to be confirmed as the inhibitory neurotransmitter in the

crustacean peripheral nervous system.

Both GABA and GAD are found in the central and peripheral parts of the mammalian nervous system (Wu and Roberts, 1974). GABA is stored in presynaptic nerve terminals and it has been shown to be released in a Ca-dependent manner (Iversen, Mitchell, and Srinivasan, 1971). Most of the GABA released following depolarization is re-accumulated by a sodium-dependent, high affinity uptake system located on nerve terminals and glia (Iversen and Neal, 1968). After its removal from the synapse by uptake into neuronal and glial cells, GABA is metabolised to succinic semialdehyde by GABA transaminase (GABA-T) (Schousboe, Wu and Roberts, 1974).

The functional significance of these biochemical events is indicated by the fact that agents which inhibit GAD, significantly reduce brain GABA levels and decrease seizure threshold (Sawaya, Horton and Meldrum, 1978). On the other hand, drugs which inhibit GABA-T increase the GABA content, depress central nervous system activity and raise the seizure threshold (Meldrum, 1978). Also, drugs inhibiting GABA transport are central nervous system depressants and provide protection against chemically induced seizures (Brehm, Krogsgaard-Larsen and Jacobsen, 1979). These results support the notion that GABA is an important inhibitory transmitter in the brain.



### 1.3 DISTRIBUTION OF GABA<sub>A</sub> RECEPTORS IN THE CENTRAL NERVOUS SYSTEM

Electrophysiological and radioligand binding studies have shown that GABA<sub>A</sub> sites are present in virtually all areas of the central nervous system including the retina and spinal cord. Peck, Schaeffer, and Clark, (1973) described the first specific binding of [<sup>3</sup>H] labelled GABA to brain tissue in synaptosomal membranes from rat cerebellar cortex

Compounds able to mimic the physiological effects of GABA (eg the toxin muscimol [3-hydroxy-5-aminomethylisoxalole]) are able to displace GABA from binding sites in membrane preparations (Greenlee, Van-Ness and Olsen, 1978). The antagonist, bicuculline, is also able to displace [<sup>3</sup>H] GABA while picrotoxin, which is presumed to interact at or near the ionophore site on the GABA receptor complex (Simmonds, 1980), does not displace [<sup>3</sup>H] GABA. Specific Na<sup>+</sup> independent binding of [<sup>3</sup>H] GABA or [<sup>3</sup>H] muscimol (a GABA agonist) to membrane fractions of rat brain was greatest in cerebellum, least in spinal cord and medulla oblongata and of intermediate potency in several other regions (Horng and Wong, 1979). An analysis of Na<sup>+</sup> independent binding to low and high affinity sites of rat central nervous system regions is shown in Table 1.0 (obtained from Placheta and Karobath, 1979).

In general, 2 separate sets of constants have been determined for the binding of [<sup>3</sup>H] GABA or [<sup>3</sup>H] muscimol, which perhaps indicate that different populations of GABA receptors exist in the mammalian central nervous system. Caution has to be taken in terms of the location of these sites, in that they could represent pre-, post- or non-synaptic (eg axonal or glial sites) GABA receptors. Further, it is not known

Table 1.0

The analysis of [ $^3\text{H}$ ] GABA binding to different regions of the rat central nervous system from Placheta and Karobath (1979).

$K_{d1}$  represents the dissociation constant for the first binding site and  $B_{\text{max}1}$  represents the density of these sites.  $K_{d2}$  represents the dissociation constant of the second binding site and  $B_{\text{max}2}$  represents the density of these sites.

CNS REGION	[ <sup>3</sup> H] GABA BINDING			
	K <sub>d1</sub>	K <sub>d2</sub>	B <sub>max1</sub>	B <sub>max2</sub>
	(nM)	(nM)	(pmol/g wet wt.)	
Cerebellum	9.2	90	130	260
Frontal Cortex	9.4	58	94	180
Olfactory bulb	13.3	78	92	165
Hippocampus	12.2	84	45	68
Striatum	9.6	73	46	110
Midbrain	16.6	58	62	106
Medulla pons	14.0	76	20	46
Spinal cord	10.8	47	9	18

whether the different types of binding sites exist as different populations in vivo, or whether they represent modification of the receptor complex caused by the binding assay.

#### 1.4 INHIBITORY ACTION OF GABA IN VERTEBRATES

The first clear demonstration that GABA has an inhibitory action on mammalian neurones came with the development of the microionophoretic technique for applying drugs locally, whilst recording from a single neurone (Curtis and Eccles, 1958). Krnjevic and Schwartz (1967) found that iontophoretically applied GABA caused hyperpolarization and lowered membrane resistance in cat cerebral cortical neurones. This response was identical to the inhibitory postsynaptic potentials evoked by surface stimulation, suggesting that GABA is an inhibitory transmitter in the cerebral cortex. The GABA mediated hyperpolarization was reversed by intracellular injection of  $\text{Cl}^-$  ions, therefore it appears to act by increasing  $\text{Cl}^-$  flux to produce membrane hyperpolarization. In 1974, Krnjevic observed a similar hyperpolarization in cat spinal motoneurones.

The hyperpolarization and the conductance increase both might be expected to contribute to inhibition; the hyperpolarization by moving the membrane potential away from the threshold for neuronal firing. The conductance increase would tend to clamp the membrane potential close to the  $\text{Cl}^-$  equilibrium potential. In most neurones, GABA produces a hyperpolarization, but a depolarization occurs in particular types of neurones, eg the primary sensory neurones and sympathetic ganglion cells. This depolarization is brought about by a

high chloride concentration within these cells, hence  $E_{Cl}$  is generally more positive (depolarized) than the resting membrane potential. Activation of GABA receptors in these cells would lead to a depolarization.

DeGroat (1972), described a picrotoxin and bicuculline sensitive GABA depolarization in neurones of the nodose ganglion. The GABA receptors on autonomic and sensory neurones are non-synaptic, GABA depolarized these neurones and this was associated with an efflux of chloride ions (Adams and Brown, 1975; Gallagher, Higashi and Nishi, 1978; Brown and Higgins, 1979). Such GABA-evoked depolarization is primarily due to the existence of a different electrochemical chloride gradient, while the intracellular chloride concentration in most central neurones is considerably lower than the extracellular one. An inwardly directed chloride pump mechanism results in a smaller concentration gradient in peripheral neurones. Studies with chloride pump inhibitors in cat dorsal root ganglion (DRG) neurones (Gallagher, Nakamura, and Shinnick-Gallagher, 1983) support the presence of an inwardly directed chloride pump. The existence of such pumps (inward and outwardly directing) has recently been demonstrated in guinea pig hippocampal neurones with frusemide, a chloride transport blocker (Misgeld, Deiss, Dodt, and Lux, 1986). Gallagher et al. (1978) estimated an internal chloride concentration of 53mM from the GABA reversal potential (assuming  $E_{Cl} = E_{GABA}$ ) in cat DRG neurones. Internal chloride concentrations are thought to be in the region of 30-60mM in peripheral neurones.

GABA was found to depolarize primary afferent nerve terminals in the spinal cord, resulting in a decrease in transmitter release (Schmidt, 1971). This depolarization and the underlying chloride conductance increase reduces the amplitude of action potentials and may inhibit their active propagation into the fine terminal arborization. Reduction of action potential amplitude, partial failure of action-potential invasion of terminal arborization ("branch point failure") and perhaps GABA actions unrelated to chloride conductance may contribute jointly in depressing transmitter release from primary afferent endings. A relatively high internal chloride concentration is a general property of peripheral neurones. This property results in the nervous system being able to use the same transmitter, in one case to hyperpolarize central neurones and in the other to depolarize the terminals of peripheral sensory neurones. The GABA receptors and associated anion channels through which GABA mediates pre- and post-synaptic inhibition appear to be similar if not identical.

### 1.5 GABA<sub>B</sub> RECEPTORS

Originally, it was thought that whenever GABA produced a change in the membrane conductance of a mammalian neurone, the ion channel associated with the GABA recognition site was predominantly chloride. However, Bowery, Hill, Hudson, Doble, Middlemiss, Shaw, Turnbull in 1980 using mammalian central and peripheral tissues described a different mammalian GABA receptor complex. This new GABA receptor complex was bicuculline (GABA<sub>A</sub> antagonist) resistant and did not

to the resting membrane conductance (Hill and Bowery, 1981). They termed this receptor the GABA<sub>B</sub> receptor to discriminate it from the classical GABA<sub>A</sub> receptor, which is coupled to a chloride ion channel and is bicuculline sensitive. A selective agonist for the GABA<sub>B</sub> receptor is baclofen (para-chlorophenyl GABA), this receptor is usually associated with cationic channels.

Newberry and Nicoll (1984) showed that (-) baclofen acting on bicuculline resistant, postsynaptic GABA receptors located on the soma and dendrites of rat hippocampal CA1 pyramidal neurones, caused a slow hyperpolarization. This effect was shown to be associated with an increase in potassium conductance. A similar action was observed in rat locus coeruleus neurones (Osmanovic and Shefner, 1986; 1988).

GABA has also been reported to decrease calcium dependent action potentials in cultured chick DRG neurones with sodium channels blocked by tetrodotoxin (Dunlap and Fischbach, 1978, 1981). In cultured rat DRG neurones, baclofen decreased the voltage dependent calcium current (Dolphin and Scott, 1986; Cottrell and Green, 1987, 1988). A similar effect of baclofen on the calcium current of cat DRG neurones *in vitro* has been shown (Robertson and Taylor, 1986). The search for a GABA<sub>B</sub> antagonist has resulted in the discovery of phaclofen (Kerr, Ong, Prager, Cynther and Curtis, 1987). This compound has a relatively low affinity (high  $\mu\text{M}$  range) for the GABA<sub>B</sub> receptor, and its specificity has yet to be characterized fully in electrophysiological tests.

Enna and Karbon (1987) proposed that the GABA<sub>B</sub> receptor acts by regulating cAMP production that is stimulated by other receptors, because GABA<sub>B</sub> receptor activation by itself does not cause cAMP production. It has been suggested that the GABA<sub>B</sub> receptor acts via one of two mechanisms. One is associated with phospholipase A<sub>2</sub> (a calcium activated enzyme that catalyzes the release of arachidonic acid from membrane phospholipids) and the other with phospholipase C which catalyzes the conversion of phosphatidyl inositol 4-5 biphosphate to the second messengers inositol trisphosphate and diacylglycerol.

Phospholipase A<sub>2</sub> liberates intracellular stores of calcium and phospholipase C stimulates protein kinase C, which phosphorylates various substrates, thereby modifying cellular activity. A role of G-proteins has been implicated in GABA<sub>B</sub> transduction but the exact role it plays remains unknown (for review see Dunlap, Holz, and Rane, 1987). For the acetylcholine muscarinic response in atrial cells, a direct action for G proteins has been shown on potassium channel activity using inside-out patches (Yatani, Codina, Brown, and Birnbaumer, 1987). It remains to be seen whether the GABA<sub>B</sub> mediated effects on calcium and potassium currents occur directly through G proteins or via some enzyme system.



## 1.6 GABA<sub>A</sub> RECEPTORS

The GABA<sub>A</sub> receptor can be described in terms of the drugs that interact with it ie the agonists and competitive antagonists. Bicuculline, an alkaloid, obtained from plants of the genera *Corydalis* and *Dicentra* was recognized as an antagonist of GABA at the GABA receptor site (Curtis, Duggan, Felix, and Johnston, 1970). Bicuculline has been shown to be a competitive antagonist within neurones of the rat cuneate slice (Simmonds, 1980) and in mouse spinal and cortical neurones (Nowak, Young and MacDonald, 1982).

Picrotoxin, a non nitrogenous polycyclic lactone, found in the plants of the *Menispermaceae* family is made up of picrotoxinin and picrotin in an equimolar ratio. Picrotoxin inhibited GABA responses non-competitively suggesting a different site of action from bicuculline (Takeuchi and Takeuchi, 1969; Simmonds, 1982; Akaike Hattori, Oomura and Carpenter, 1985b). Binding studies using radioactive dihydropicrotoxinin indicate that picrotoxin acts on GABA-activated chloride channels rather than on GABA recognition sites (Olsen, 1981). Recently, a new ligand [<sup>35</sup>S]-t-butyl-bicyclophosphorothionate (TBPS) has been introduced to study binding sites associated with the GABA-activated chloride channel (Squires, Casida, Richardson and Saederup, 1983). TBPS is a convulsant and acts as a GABA antagonist through an interaction with the chloride channel (Olsen, 1981).

## 1.7 BENZODIAZEPINE RECEPTORS

The benzodiazepines are a very interesting class of drugs with unique clinical properties. They are extremely effective in alleviating anxiety and also have uses as anticonvulsants and muscle relaxants (Tallman and Gallagher, 1985). As with many psychoactive drugs, the mechanism of benzodiazepine action has remained unclear even though their clinical use has been widespread. Recently studies have focussed on the interrelationship with GABAergic neurones. The first reports of specific (displaceable), saturable, high affinity binding sites for benzodiazepines appeared in 1977 (Braestrup and Squires, 1977; Mohler and Okada, 1977). [ $^3\text{H}$ ] Diazepam binding not only occurred in brain tissue, but also in peripheral tissues such as the liver and the kidney.

This specific recognition site for the benzodiazepines provided a point of focus for subsequent investigation. In 1978, Tallman, Thomas and Gallagher using binding studies in cortical membranes observed enhanced binding of [ $^3\text{H}$ ] diazepam when either GABA or its analogue muscimol was included in the in vitro assay for benzodiazepine receptors. This observation suggested for the first time a functional interaction between the GABA<sub>A</sub> receptor and the benzodiazepine binding site (the benzodiazepine [BZ] receptor). Further evidence was obtained of an interaction between BZ receptors and GABA receptors (Schoch, Richards, Haring, Takacs, Stahl, Staehelin, Haefely and Mohler, 1985). These workers using monoclonal antibodies showed co-localization of GABA<sub>A</sub> and benzodiazepine receptors in the rat brain.

### 1.7.1 PERIPHERAL TYPE BENZODIAZEPINE RECEPTOR

As well as [<sup>3</sup>H] diazepam binding to brain tissue, Braestrup and Squires (1977) also observed binding to peripheral tissues such as the lung and kidney. This kidney(peripheral) type benzodiazepine binding site has a completely different pharmacological profile from that of the central (brain) type receptor. Relative potencies of benzodiazepines at the central BZ sites closely parallel their therapeutic efficacy as anticonvulsants and anti-anxiety agents. However, some of the most potent benzodiazepines at the central site, such as clonazepam, are a thousand times weaker at binding to the peripheral BZ site.

Benzodiazepines such as Ro5-4864 (a drug synthesized by Hoffman La-Roche) display high ( $K_d$ -low nM range) affinity for the peripheral binding sites but are less than 0.1% as potent at central receptors. In spite of the name peripheral benzodiazepine receptors, receptors displaying the peripheral type pharmacology can be identified in the central nervous system using peripheral type specificity ligands [<sup>3</sup>H] Ro5-4864. Autoradiography of rat brain regions reveals peripheral benzodiazepine sites selectively concentrated in the ependyma, choroid plexus and olfactory bulbs (Anholt, Murphy, Mack, and Snyder, 1984). The exact physiological role of the peripheral type BZ receptor remains to be determined, however, recent subcellular localization studies have revealed a selective association with the outer mitochondrial membrane. A possible link with voltage dependent anion channels has been proposed along with porphyrin as being the endogenous ligand for this receptor (for review see, Snyder, Verma, and Trifiletti, 1987).

Parenterally administered Ro5-4864 has recently been reported to be a convulsant in guinea pigs, rat and mice with  $CD_{50}$  values of 17, 12.8 and 23.5mg/kg respectively (Weissman, Colt, Hommer, Paul and Skolnick, 1984). These convulsions were not prevented by the benzodiazepine antagonist Ro15-1788 suggesting that this effect was not related to a direct interaction with central BZ receptors. In neurones of the rat cuneate nucleus slice preparation, Ro5-4864 (0.1 $\mu$ M) antagonized the potentiating action of flurazepam on muscimol responses. At higher concentrations Ro5-4864 (3 $\mu$ M) enhanced the effect of picrotoxin and antagonized the pentobarbitone induced enhancement of muscimol responses (Pellow, File and Simmonds, 1984).

Ro5-4864 (>1 $\mu$ M) also inhibited GABA responses non-competitively in cultured mouse spinal neurones (Skerritt, Wertz, Mclean and MacDonald, 1984). In radioligand binding studies, Ticku and Ramanjaneyula (1984) have shown that Ro5-4864 inhibits [ $^{35}$ S] TBPS binding at the concentrations at which it produces convulsions. These results indicate that Ro5-4864 acts on a low affinity site of the GABA-receptor complex that is close to or identical to the picrotoxin binding site.

### 1.7.2 CENTRAL TYPE BENZODIAZEPINE RECEPTOR

Three different type of ligands can interact with the central BZ receptor, each with different functional effects (Richards, Schoch, Mohler and Haefely, 1986).

#### AGONISTS

Benzodiazepine agonists have anxiolytic, hypnotic, anticonvulsant and muscle relaxant properties. They enhance the chloride conductance thereby increasing the inhibitory effect of GABA. Benzodiazepine agonists include diazepam, alprazolam, triazolam, flunitrazepam, and clonazepam.

#### INVERSE AGONISTS

The term inverse agonist has been used for ligands which act at the benzodiazepine site but have an action opposite to that of agonists such as diazepam. They reduce the effect of GABA and are anxiogenic and convulsant. Most of the inverse agonists are  $\beta$ -carbolines for example  $\beta$ -carboline-3-carboxylate-ethyl ester ( $\beta$ -CCE) the methyl ester ( $\beta$ -CCM) and methyl-6-7-dimethoxy-4-ethyl  $\beta$ -carboline-3-carboxylate (DMCM).

#### COMPETITIVE ANTAGONISTS

Competitive antagonists are practically inactive per se but prevent or abolish the receptor mediated pharmacological or therapeutic effects of agonists and inverse agonists. Examples of antagonists include CGS 8216 and the imadazodiazepine compound Ro15-1788. Ro15-1788 has proved very useful in determining whether or not certain drugs that can affect GABAergic transmission are acting at

the central BZ site.

### 1.7.3 POSSIBLE SUBTYPES OF THE CENTRAL BENZODIAZEPINE RECEPTOR

Recently two subtypes of the central BZ receptor have been proposed. The triazolopyridazine anxiolytics CL 218872 and some  $\beta$ -carboline-3-carboxylate esters inhibit benzodiazepine binding in a biphasic manner (Nielsen and Braestrup, 1980). Both types of ligand bind with greater affinity to a receptor subtype that predominates in the cerebellum (termed BZ<sub>1</sub>). The BZ<sub>1</sub> sites show a 10 fold higher affinity for  $\beta$ -carbolines and triazolopyridazines than the BZ<sub>2</sub> sites. Benzodiazepines such as diazepam show very similar affinities for the two subtypes and are therefore unable to distinguish between them. The two subpopulations appear to be 2 different proteins on the basis of ontogeny regional localization (Young, Neihoff, Kuhar, Beer and Lippa, 1981), detergent solubilization properties and sensitivity to modulation by ions (Lo, Strittmatter and Snyder, 1982).

Several workers (Gee, Erhlert and Yamamura, 1983; Braestrup, Schmichen, Noff, Nielsen and Petersen, 1982) interpret the apparent heterogeneity of benzodiazepine receptors on the basis of different allosteric states of a single receptor protein modulated by ligands differing in efficacy. This notion has gained further credence by Sato and Neale (1987) using 2 dimensional electrophoresis of the benzodiazepine receptors from rat cerebellum (90% Type 1) and cortex (mixture of type 1 and 2). Similar subunit proteins were found from these two regions suggesting that they represent different conformations of the same complex. The idea of receptor subtypes is

an attractive hypothesis in terms of function, where it has been speculated that there should be a benzodiazepine receptor which mediates anxiolytic mechanisms and one which mediates the sedative properties of the benzodiazepines (Braestrup and Nielsen, 1982).

#### 1.7.4 ACTIONS OF BENZODIAZEPINES ON MEMBRANE CURRENTS

At around the same time as specific binding sites for benzodiazepines were first being demonstrated, insights into their mechanisms of action were being made. Benzodiazepines have been shown to enhance the physiological response to GABA and to potentiate inhibitory postsynaptic potentials (ipsp) at synapses where GABA is thought to be the natural transmitter (Polc and Haefely, 1976; Choi, Farb, and Fischbach, 1977). Midazolam potentiated the depolarizing action of GABA on primary afferent endings in the frog hemisectioned spinal cord preparation (Nistri and Berti, 1984). Little, also in 1984, described potentiating actions of chlordiazepoxide and flurazepam on GABA responses in the rat superior cervical ganglion.

Chlordiazepoxide selectively potentiated GABA responses in cultured chick spinal and sensory neurones (Choi, Farb and Fischbach, 1981), causing a concentration dependent left hand shift of the GABA dose-response curve. Nicoll and Waltowicz (1980) also observed an enhancement in the GABA-evoked hyperpolarization with chlordiazepoxide in frog spinal motoneurones; Nistri, Constanti and Krnjevic (1980) obtained similar results with flurazepam in cat spinal motoneurones *in situ*. Iontophoresized midazolam and flurazepam potentiated GABA responses in the CA1, CA3, and dentate cells of the mouse hippocampal



slice (Biscoe and Duchon, 1985). These benzodiazepine agonists potentiated GABA responses in 70% of the cells tested, 15% showed a decrease, while the remaining 15% showed no detectable response to these drugs.

Diazepam (1-10 $\mu$ M) has also been reported to increase GABA-evoked whole-cell currents in the isolated bovine adrenal medullary cell (Cottrell, Lambert and Peters, 1985; Bormann and Clapham, 1985). Inhibitory postsynaptic currents (ipscs) evoked by GABA in cultured neonatal rat cortex were increased by flunitrazepam and decreased by the inverse agonist DMCM. These actions were blocked by the BZ receptor antagonist Ro15-1788; 20-30% of the cells tested in this study showed no response to these drugs (Vicini, Alho, Costa, Mienville, Santi, and Vaccarin, 1986). The effect of diazepam on GABA-activated chloride conductances in internally perfused frog sensory neurones was investigated by Hattori, Oomura and Akaike (1986). A bell shaped dose-response curve was described with maximum enhancement of the GABA current occurring at a diazepam concentration of 10 $\mu$ M. Diazepam potentiated pentobarbitone enhanced GABA currents indicating different sites of actions for these two drugs on the GABA<sub>A</sub> receptor.

One recently described important in vitro assay, is the flux of radioactive [<sup>36</sup>Cl<sup>-</sup>] in various brain tissues; this technique allows a biochemical and pharmacological assay of GABA receptor function at the cellular level (Wong, Leeb-Lundberg, Teichberg, and Olsen, 1984). Several workers have used this technique to examine the effects of benzodiazepines on GABA stimulated [<sup>36</sup>Cl<sup>-</sup>] flux. Yang and Olsen (1987) showed that flunitrazepam enhanced the [<sup>36</sup>Cl<sup>-</sup>] flux stimulated



by muscimol in mouse cortical slices but did not have any action on its own. The agonists diazepam, flunitrazepam and clonazepam potentiated GABA stimulated [ $^{36}\text{Cl}^-$ ] uptake in membrane vesicles from the rat cerebral cortex, whereas the inverse agonists  $\beta$ -CCM,  $\beta$ -CCE and DMCM inhibited [ $^{36}\text{Cl}^-$ ] uptake. These effects were blocked by Ro15-1788 (Obata and Yamamura, 1987)

Benzodiazepine agonists (clonazepam, diazepam, and flurazepam) enhanced [ $^{36}\text{Cl}^-$ ] influx, along with  $\beta$ -CCPr, but DMCM attenuated [ $^{36}\text{Cl}^-$ ] influx in cultured mouse spinal neurones (Lehoullier and Ticku, 1987). A recently synthesized imadazobenzodiazepine (Ro 15-4513) has been reported to antagonize ethanol stimulated [ $^{36}\text{Cl}^-$ ] uptake into brain vesicles (Suzdak, Glowa, Crawley, Schwartz, Skolnick, and Paul, 1986). Ro15-4513 has now been shown to increase firing of neurones within the substantia nigra, this increase was prevented by Ro15-1788 (Mereu, Passino, Carcangia, Boi, and LuigiGessa, 1987). These authors propose that Ro15-4513 is an inverse BZ agonist. These studies suggest that some of the central nervous system effects of ethanol may be mediated by an interaction with GABA-BZ receptors. Ro15-4513 is currently being investigated clinically as an agent capable of reversing the effects of ethanol. In future, [ $^{36}\text{Cl}^-$ ] flux assays will play an important role in the screening of potential new drugs that affect the GABA<sub>A</sub> receptor.

Skovgaard Jensen and Lambert (1986) using intracellular recording on cultured mouse spinal neurones investigated the actions of the agonist midazolam and the inverse agonist DMCM. Midazolam (>1 $\mu\text{M}$ ) reliably potentiated GABA responses with a parallel shift to the left of the dose-response curve. DMCM (>1 $\mu\text{M}$ ) reduced the GABA responses in

a non-competitive manner. Skerritt and MacDonald (1984a) described potentiating actions of the agonists, diazepam, clonazepam and nitrazepam on GABA responses in cultured mouse spinal neurones. These actions occurred with agonist concentrations in the low nM range. These authors in an accompanying report (Skerritt and MacDonald, 1984b) also examined the actions of the  $\beta$ -carbolines on the GABA responses. DMCM reduced the GABA response but  $\beta$ -CCPr enhanced GABA responses in a concentration dependent manner, this correlated well with the chloride flux assays of Lehoullier and Ticku (1987).

Triazolam is a short acting drug used clinically in the treatment of insomnia, in association with anxiety states (Clow, Glover, and Sandler, 1985). The effect of triazolam on GABA responses in cultured mouse spinal neurones was examined by Mathers and Yoshida (1987). Triazolam had both direct and GABA depressant actions on these neurones. Triazolam (1 $\mu$ M) caused a direct depolarization in 50% of the neurones studied and this drug also attenuated GABA evoked depolarizations in 50% of the neurones. GABA-activated single channel current activity in outside-out patches was decreased by triazolam. The anxiolytic effects of this drug appears not to be attributed to an action on the GABA-BZ receptor since its actions appear to be more typical of an inverse agonist.

The potentiation of GABA action by benzodiazepine receptor agonists appears to be fairly selective. Neither the monoamines nor the other established putative inhibitory amino-acid neurotransmitters (glycine,  $\beta$ -alanine, and taurine) were affected by benzodiazepines in concentrations that increase the effect of GABA (Choi, Farb and Fischbach, 1981; Skerritt, Wertz, Mclean and MacDonald, 1984). One

report, however, has shown that diazepam (1 $\mu$ M) can potentiate the action of GABA analogues and other transmitters such as glycine,  $\beta$ -alanine and taurine in frog DRG neurones (Hattori, Oomura, and Akaike, 1986). Benzodiazepine receptor agonists have been reported to have no effect on excitatory neurotransmitters (Skerritt et al., 1984a).

Some reports have been made as to effects of benzodiazepine agonists on neuronal membrane properties quite distinct from their actions on the GABA<sub>A</sub> receptor. Carlen, Gurevich and Polo in 1983 described an enhancement in a calcium dependent potassium conductance in hippocampal pyramidal neurones with the agonist midazolam (1-10nM). The agonist flurazepam (1pM-10nM) had multiple actions on cultured mouse spinal neurones, apart from potentiating responses to GABA, it also directly evoked a membrane conductance to chloride ions, elevated spike threshold, and depressed repetitive spike firing (MacDonald and Barker, 1982).

Cardiovascular actions of diazepam including its negative inotropic and chronotropic properties, vasodilating and anti-arrhythmic activities have suggested a possible involvement of central benzodiazepines with calcium channels (for review see, Rampe and Triggle, 1986). A study by Johansen, Taft, Yang, Kleinhaus, and Delorenzo (1985) suggested that benzodiazepines may block calcium channels in neurones. This interaction with calcium channels was later confirmed by Watabe, Yoshii, Ogata, and Narahashi (1986) using patch clamp studies on a mouse neuroblastoma cell-line N1E-115. Diazepam (100 $\mu$ M) and nitrazepam reduced a type I and type II barium current while clonazepam (100 $\mu$ M) reduced the type I current

preferentially. Type I (transient) currents are activated by a voltage step to potentials greater than -50mV, while type II currents are activated by a step to potentials greater than -20mV. The concentration of benzodiazepine agonists exerting these effects are some 10-100 times higher than that causing GABA potentiation, it remains to be seen whether these actions of benzodiazepines on calcium channels are of physiological significance.

#### 1.7.5 MOLECULAR MECHANISM OF ACTION OF BENZODIAZEPINES

Benzodiazepine agonists could exert their modulatory action on GABA responses via a number of different mechanisms. Some of these include

- (1) an increase in the conductivity of the GABA-activated channels
- (2) a change in the ion selectivity of the channels or
- (3) an increase of the affinity of the receptors for GABA.

The mechanism of action of benzodiazepines was investigated using fluctuation (noise) analysis of the GABA-induced membrane current noise (the random miniature jumps in the current flow representing single channel gating events) in voltage clamped cultured mouse spinal neurones (Study and Barker, 1981). These studies showed that diazepam increased the probability of single channel opening events with no change in the single channel conductance and little, if any increase in the mean single channel open time. On the other hand,  $\beta$ -CCE and DMCM reduced the frequency of single channel opening events without affecting the average channel lifetime (Barker, Gratz, Owen and Study, 1984).

The most direct approach to the study of the molecular mechanism of action of benzodiazepines has been the recording of single GABA gated chloride channels in excised patches from spinal cord neurones. A preliminary report by Bormann and Sakmann (1984) indicated that the only effect of diazepam was to increase the frequency of channel openings in response to GABA. Similar observations were described by Rogers and MacDonald (1986) but these workers also reported an increase in the burst duration with 250nM diazepam.

The single channel response to GABA in the presence of benzodiazepine agonists was found to be similar to a higher concentration of GABA in the absence of the drug. These findings suggest that either (i) an increase in the GABA receptor affinity, (ii) a facilitated GABA receptor activation, (iii) a facilitated gating function of the receptor-ionophore complex or (iv) any combination of these three individual mechanisms may account for the potentiation of GABA by BZ agonists. In general, benzodiazepine agonists seem to increase the sensitivity of the GABA receptor ionophore function. In the presence of an agonist less GABA is required to induce a given chloride conductance than in its absence.

#### 1.7.6 ENDOGENOUS BENZODIAZEPINE LIGANDS

Since the existence of specific receptors for the benzodiazepines (Mohler and Okada, 1977) it has been suggested that an endogenous ligand may exist for this receptor. This ligand could possess either anxiolytic or anxiogenic properties. To date 3 endogenous ligands are being proposed by various workers.

The first concerns the presence of n-butyl- $\beta$ -carboline-3-carboxylate ( $\beta$ -CCB) which has recently been isolated from the bovine cerebral cortex (Pena, Medina, Nova, Paladini and Robertis, 1986). Previous successful attempts in the isolation of the ethyl ester concluded that the material was an extraction artifact (Braestrup, Nielsen and Olsen, 1980). This recent study took considerable pains to eliminate this possibility. The methyl and ethyl esters of  $\beta$ -carboline-3-carboxylate are anxiogenic. The tert-butyl  $\beta$ -carboline-3-carboxylate increased the frequency and open time kinetics of the GABA channel in a manner similar to diazepam and other agonists (Owen, Study, Gratz and Barker, 1982). Therefore the  $\beta$ -CCB could be an agonist at the BZ site and be an anxiolytic, however, thorough behavioural studies have yet to confirm this.

A rather different approach employing monoclonal antibodies has been used by Deblas, Sangameswaram, Hanley, Park, Abraham and Raynor (1985). Antibodies were selected for an epitope associated with the benzodiazepine skeleton. These antibodies were then used to localise immunologically active material in mammalian brain. A substance N-desmethyldiazepam (nordiazepam) was isolated from human brain using various procedures including HPLC, spectrophotometry, binding studies



and immunological techniques. BZ like immunoreactivity was also found in human brains from the year 1940 prior to the first synthesis of benzodiazepines (1955) suggesting that they were of natural origin (Sangameswaram, Fales, Friedrich and Deblas, 1986). Since then BZ like immunoreactivity has been located in peripheral tissues such as the liver, kidney and spleen and has been found in the cytoplasmic face of the neuronal membrane. These authors suggest that the BZ like molecules might be involved in modulating GABAergic transmission and/or the biotransformation, accumulation and elimination of BZ and BZ-like molecules in the brain (Deblas and Sotelo, 1987; Deblas, Park and Friedrich, 1987).

The third candidate for the endogenous ligand is the one of longest standing. This substance was originally isolated in an impure form in 1978 by Guidotti, Toffano and Costa. Since its original discovery, considerable advances in the characterization of this molecule have been made by this group. This molecule is a polypeptide called Diazepam Binding Inhibitor (DBI) because it can competitively displace specific ligands from the benzodiazepine recognition site. DBI is 105 amino-acids long and has a molecular weight around 11,000. Proteolytic digestion of DBI revealed an 18 amino-acid octodecanuropeptide (ODN) peptide with increased biological activity (Ferrero, Santi, Conti-Tronconi, Costa and Guidotti, 1985).

When tested in a behavioural model of anxiety in rats, DBI elicited a proconflict (enhanced the shock induced suppression of drinking) action when injected intraventricularly (Guidotti, Forchetti, Korda, Kankel, Bennetti and Costa, 1983). Recently DBI has been shown to have a subcellular localization and to be co-released

along with GABA upon depolarization. Double immunofluorescence staining of cultured cerebral cortex neurones of the neonatal rat with GAD and antibodies for ODN, indicated that ODN and ODN like peptides are localized with GABA in 58% of the GAD-positive neurones (Ferrarese, Alho, Guidotti, and Costa, 1987). These results suggest that ODN, or ODN like peptides derived from DBI might participate as putative neuromodulators of physiological significance in GABAergic neurotransmission.

DBI's anxiogenic profile is supported by electrophysiological studies in which DBI alone had no effect on the GABA<sub>A</sub> receptor but 10 $\mu$ M DBI reduced GABA-evoked chloride currents by 42% in cultured mouse spinal neurones. This reduction was blocked by Ro15-1788 (Bormann, Ferrero, Guidotti and Costa, 1985). The exact molecular mechanism of DBI's action still needs to be determined, but this effect is very similar to that observed for  $\beta$ -carbolines (Skerritt and MacDonald, 1984b).

## 1.8 BARBITURATES

Barbiturates have been used in clinical medicine for over 80 years. The discovery of the benzodiazepines superseded the general use of barbiturates as sedative-hypnotic agents, but there is still an important therapeutic role for barbiturates as anaesthetics and anticonvulsants. Barbiturates have been shown to have both pre- and post-synaptic actions on a variety of synapses and neurotransmitter systems, to influence axonal properties, mitochondrial function, uptake and release of neurotransmitters and ion transport (for review



see, Willow and Johnston, 1983).

There are several ways in which the cellular effects of barbiturates could lead to generalised depression of neuronal excitability and general anaesthesia.

- (a) Selective postsynaptic depression of glutamate excitation.
- (b) Prolongation of GABA mediated postsynaptic inhibition.
- (c) Prolongation of GABA mediated presynaptic inhibition leading to partial deafferentation.
- (d) Direct effects on CNS neurones to depress their excitability.

The relative importance of each of these in producing general anaesthesia still remains to be determined.

#### 1.8.1 RADIOLIGAND BINDING STUDIES OF BARBITURATES

Barbiturates have several different actions on the GABA<sub>A</sub> receptor, they have been shown to enhance the binding of GABA in both chloride dependent and independent manners, to alter the interaction of dihydropicrotoxin and also to enhance benzodiazepine binding. In all of these actions, barbiturates display considerable structural specificity suggestive of the involvement of specific barbiturate binding sites.

The chloride dependent enhancement of binding of GABA and its analogues has been reported by several groups (Asano and Ogasawara, 1982; Olsen and Snowman, 1982; Whittle and Turner, 1982). The enhancement of GABA binding by barbiturates was blocked by the

ionophore antagonist picrotoxin (Olsen and Snowman, 1982) and was not affected by Ro15-1788. Chloride independent enhancement of GABA binding by pentobarbitone has also been described (Asano and Ogasawara, 1981). Sedative, hypnotic and anticonvulsant barbiturates inhibit the binding of dihydropicrotoxin to brain membranes (Ticku and Olsen, 1978). Barbiturates such as pentobarbitone enhance benzodiazepine binding via an increase in affinity involving predominantly a decreased rate of dissociation (Ticku, 1981). It is interesting that several barbiturates such as phenobarbitone and non-barbiturates (eg chlorometriazole) which are used clinically as anticonvulsants do not enhance diazepam binding. However, these compounds inhibit the enhancing effect of barbiturates like pentobarbitone, indicating a different kind of interaction with the same site (Leeb-Lundberg and Olsen, 1982).

The barbiturate recognition site on the GABA/benzodiazepine receptor complex is distinct from the picrotoxinin/TBPS recognition site but they do appear to be allosterically linked (Trifiletti, Snowman, and Snyder, 1985). These results suggest that while barbiturates bind to the GABA<sub>A</sub> receptor complex at sites near the chloride ionophore, benzodiazepines act at separate sites to influence GABA<sub>A</sub> receptors (Johnston and Skerritt, 1983).

### 1.8.2 ACTIONS OF BARBITURATES ON MEMBRANE CURRENTS

The first electrophysiological observation relevant to the interaction of barbiturates with GABA receptors was made by Eccles and Malcolm (1946) who found that pentobarbitone prolonged the decay of the dorsal root potential in the isolated frog cord. Increases in dorsal root potential are now considered to reflect a reduced release of primary afferent transmitter, initiated by a depolarizing action of GABA (Levy, 1977). The enhancement by barbiturates of both pre- and post-synaptic inhibition mediated by GABA has been observed in many electrophysiological studies and in addition some barbiturates (notably pentobarbitone) may have a direct agonist like action on the GABA<sub>A</sub> receptor.

In cultured mouse spinal neurones iontophoretic application of pentobarbitone (Ransom and Barker, 1975) prolonged the inhibitory action of GABA and also produced a decrease in membrane resistance accompanied by hyperpolarization. Although pentobarbitone has a predominantly sedative action in vivo phenobarbitone is highly effective as an anticonvulsant producing minimal sedation. These two barbiturates have different actions in vitro on cultured spinal neurones (MacDonald and Barker, 1979). At the same concentration (200 $\mu$ M)

(a) Pentobarbitone but not phenobarbitone acted as a GABA agonist.

(b) Pentobarbitone was much more effective than phenobarbitone in suppressing spontaneous activity, but phenobarbitone effectively attenuated picrotoxin induced convulsive activity.

The relative lack of effect of phenobarbitone on spontaneous activity and membrane conductance may explain why this drug does not possess anaesthetic properties, relevant to its anticonvulsant properties.

Pentobarbitone (50-100 $\mu$ M) greatly prolonged the decay of GABA-evoked inhibitory postsynaptic currents (ipscs) in adult rat hippocampal CA1 neurones (Collingridge, Gage, and Robertson, 1984; Gage and Robertson, 1985). A similar prolongation of the ipscs was shown in cultured hippocampal neurones from the embryonic rat (Segal and Barker, 1984). Inhibitory postsynaptic potentials in rat hippocampal neurones were prolonged by barbiturates (Nicoll, Eccles, Oshima, and Rubia, 1975). GABA-evoked whole-cell currents were potentiated by 500 $\mu$ M phenobarbitone in bovine chromaffin cells (Cottrell et al., 1985).

A direct agonist like action of pentobarbitone at high concentrations (approx. 1mM) has been described by several workers (Simmonds, 1981; Higashi and Nishi, 1982; Nicoll and Madison, 1982; Akaike, Hattori, Inomata and Oomura, 1985a; and Robertson, 1986). This direct agonist action is suggested since (for review see, Owen, Barker, Segal, and Study, 1986).

(a) The dose-response curve is parallel to that of GABA with an equally steep slope suggestive of a co-operative ligand binding step.

(b) Pentobarbitone activates chloride ion channels with identical

conductance to that of GABA.

(c) The pentobarbitone action is sensitive to agents that interact with the GABA<sub>A</sub> receptor either directly (bicuculline) or allosterically (diazepam).

Similar potentiating actions of barbiturates were obtained using GABA stimulated [<sup>36</sup>Cl<sup>-</sup>] flux changes in various brain tissues (Obata, Palmar, Laird, and Yamamura, 1986; Allan and Harris, 1986; Yang and Olsen, 1987).

Barbiturates tend to be specific in that they potentiate GABA action as opposed to other inhibitory neurotransmitters. However, the actions of excitatory transmitters (glutamate, aspartate, acetylcholine) start to be depressed at concentrations similar to or only slightly higher than those causing GABA potentiation (Ransom and Barker, 1976; Barker and Ransom, 1978; MacDonald and Barker, 1978). Pentobarbitone (75-200μM) has been shown to block excitatory amino-acid (L-aspartate, kainic acid, and quisqualic acid) transmission in cultured hippocampal neurones by Mijkovic and McDonald (1986). This block was voltage-dependent and suggestive of a block of the open amino-acid activated channels.

An interaction with voltage dependent calcium channels has also been suggested (MacDonald and Wertz, 1985). These workers showed that phenobarbitone (500μM) and pentobarbitone (100μM) reduced voltage dependent calcium currents in mouse spinal neurones. Pentobarbitone primarily affected the N and L type voltage dependent calcium channels in mouse sensory neurones (Gross and MacDonald, 1986). This action of the barbiturates on calcium channels may produce sedative-hypnotic and

anaesthetic actions. This is achieved in part by a non-specific (non-selective) reduction of presynaptic calcium entry and subsequent reduction of transmitter release from synapses throughout the nervous system.

Enhancement of a potassium conductance by pentobarbitone (Carlen, Gurevich, Davies, Blaxter and Bierne, 1985) in mammalian hippocampal and cerebellar slices has been reported. Pentobarbitone (1-10 $\mu$ M) caused a hyperpolarization with a decrease in spontaneous spike activity and enhanced post spike hyperpolarizations. Similar actions were also described earlier in frog motoneurons and rat hippocampal pyramidal neurones (Nicoll and Madison, 1982). Barbiturates in radioligand binding studies are also thought to be selective antagonists at  $A_1$  Adenosine receptors (Lohse, Klatz, Jacobs and Schwabe, 1985). Barbiturates competitively inhibited the binding of agonists and antagonists to  $A_1$  receptors, however, electrophysiological confirmation of this interaction have yet to be performed.

### 1.8.3 MOLECULAR MECHANISM OF ACTION OF BARBITURATES

Fluctuation (noise) analysis techniques have shown that barbiturates have a clearly different mechanism of action from the benzodiazepine receptor agonists (Barker and McBurney, 1981). Ionophoresed phenobarbitone prolonged the duration of the unitary current fluctuations from 24-120ms without affecting the amplitude or the rise time of these currents, indicating that the principal action of phenobarbitone was to increase the average time during which the

GABA-activated channels remained open (Study and Barker, 1981). Apart from prolonging the mean open time of the GABA-activated single channels, a reduction in the frequency of single-channel openings was noted. Mathers and Barker (1980), also using fluctuation analysis showed that GABA and (-) pentobarbitone activated single chloride channels of similar conductance but very different duration. The channels activated by pentobarbitone remained open much longer than those activated by GABA. This finding was confirmed a couple of years later using both GABA and pentobarbitone in the patch pipette. Cell-attached patches obtained from cultured spinal neurones showed that the pentobarbitone-gated chloride channels were open much longer than those of GABA (Jackson, Lecar, Mathers and Barker, 1982).

The only single channel studies on the mechanism of action of barbiturates (Tyman, Rogers and MacDonald, 1987) was performed recently using outside-out patches from mouse spinal neurones. These authors showed that the enhancement of GABA-activated chloride currents by pentobarbitone and phenobarbitone was due to an increased number of channel openings; an increased duration of individual channel openings; and an increase in the burst duration.

#### 1.9 INVERTEBRATE GABA RECEPTORS

There appears to be a few differences, between the GABA receptors found in invertebrates and the GABA<sub>A</sub> receptor in vertebrates. While bicuculline is a competitive antagonist at the vertebrate GABA<sub>A</sub> receptor it is generally inactive in invertebrates. Also although chloride ions are mainly involved in GABA responses of vertebrates,



GABA operated cation selective channels have been described in invertebrates.

GABA is a neurotransmitter in many invertebrate species (Gershenfeld, 1973). GABA is thought to be the inhibitory neurotransmitter at the locust neuromuscular junction (Usherwood and Grundfest, 1965). Using the extensor-tibiae muscle of the metathoracic leg of *Schistocerca gregaria*, noise analysis techniques have shown that GABA opens ionic channels which are chloride selective and which have a conductance of 22pS (Onodera and Takeuchi, 1976). GABA is also the inhibitory transmitter in crustacea and its inhibitory action has been shown to primarily involve chloride ions (Takeuchi and Takeuchi, 1967, 1971a,b). The early electrophysiological work mostly involved voltage clamp (Dudel, 1977, 1978) and noise analysis techniques (Dudel, Finger and Stettmeier, 1980).

The patch clamp technique has now been successfully applied to different regions of crayfish muscle (Franke, Hatt and Dudel, 1986a,b). In their study of glutamate activated channels in hyperpolarized cell-attached patches, these workers described a chloride permeable channel gated by glutamate. This glutamate receptor ion-channel complex was surprisingly also found to be sensitive to GABA. These two transmitters displayed competition in activating the chloride channel in excised outside-out patches.



Small differences were noted in the types of single channel currents activated by these transmitters. The chloride channel had 3 distinct substates of 22, 44 and 66pS. Glutamate preferentially activated the 22pS state, whereas GABA preferred to activate the 44pS state, but all 3 states could be activated by each agonist at high concentrations. The glutamate analogue quisqualate and the GABA analogue guanidine-propionic acid also preferred to activate the 22 and 44pS states respectively. A permeability value of  $3.5 \times 10^{-14} \text{ cm}^3 \text{ s}^{-1}$  was obtained for the 22pS state using the single-channel I-V relationship and the chloride concentrations. The physiological significance of this ion-channel remains unknown but the authors suggest that receptor channel complexes at both excitatory and inhibitory synapses are derived from a common ancestor.

Some GABA-operated cation channels have been found in molluscs. Five different responses to GABA have been described in neurones of the *Aplysia* thoracic ganglia (Yarowsky and Carpenter, 1979) two of these are inhibitory while three are excitatory. One of the excitatory responses is associated with an increase in sodium conductance. A second is associated with increases in both sodium and chloride, while the third was possibly associated with a decrease in potassium conductance. Of the two inhibitory responses to GABA, one is associated with fast hyperpolarization and an increase in chloride conductance, while the other is associated with slow hyperpolarization and an increase in potassium conductance. These GABA receptors are pharmacologically distinct. Curare selectively blocks fast sodium and chloride channels while picrotoxin and bicuculline block only fast chloride channels. Chloride dependent responses to GABA have been

shown in neurones of the abdominal ganglia of *Aplysia kurodai* (Matsumoto, Sasaki, Schozushima and Sato, 1986).

#### 1.9.1 MODULATION OF GABA RESPONSES IN INVERTEBRATES

The antagonists bicuculline and picrotoxin have been shown to block GABA-gated chloride conductances in mammalian systems through different mechanisms (for review see Simmonds, 1983; Akaike, Hattori, Oomura and Carpenter, 1985b). Akaike et al. (1985b) showed that bicuculline competitively inhibited GABA responses in frog DRG neurones, however picrotoxin had a non-competitive action. Picrotoxin (10 $\mu$ M) reversibly blocked GABA responses from either side of the membrane, indicating an action on the chloride channel rather than on the receptor. This finding suggested that the picrotoxin binding site was at/or near the chloride channel rather than on the receptor.

Picrotoxin blocked GABA-gated chloride conductances in most invertebrate species for example in the dactyl opener muscle from the lobster (Smart and Constanti, 1986); neurones from the thoracic ganglia of *Schistocerca gregaria* and *Locusta migratoria* (Lees, Beadle, Newmann, and Benson, 1987); an identified motoneurone from the thoracic ganglia of *Periplaneta americana* (Wafford, Sattelle, Abalis, Eldefrawi and Eldefrawi, 1987), cultured neurones from the cockroach brain (Shimahara, Pichon, Lees, Beadle and Beadle, 1987) and skeletal muscle fibres of *Schistocerca gregaria* (Scott and Duce, 1987). In addition there is one report by Albert, Lingle, Marder and O'Neill (1986) where chloride responses to GABA in the gm6b and opener muscles of the spiny lobster *Panulirus interruptus* and *Panulirus argus* were

insensitive to picrotoxin as high as 50 $\mu$ M. Responses in the corresponding muscle of *Homarus americanus* could be blocked by picrotoxin at 1 $\mu$ M.

Bicuculline, which is a competitive antagonist at the vertebrate GABA<sub>A</sub> receptor is generally not an antagonist at the invertebrate GABA receptor (Scott and Duce, 1987; Smart and Constanti, 1986; Shimahara et al., 1987 and Lees et al., 1987). In neurones of the *Aplysia* thoracic ganglia chloride dependent responses to GABA and acetylcholine are both blocked by bicuculline (Yarowsky and Carpenter, 1978).

Benzodiazepine and barbiturate sites on some invertebrate GABA receptors are recently becoming apparent in radioligand binding and electrophysiological studies. This is despite the fact that diazepam binding studies on neuronal tissue from vertebrate and invertebrates, suggested that benzodiazepine receptors do not occur in the invertebrates but arose with the evolution of the bony fishes (Nielsen, Braestrup and Squires, 1978). A high affinity ( $K_d = 47$ nM) benzodiazepine binding site has been described by Robinson, Macallan, Lunt and Battersby (1986) in membranes of the supraoesophageal ganglion of the locust. This site pharmacologically appears to resemble the peripheral benzodiazepine site rather than the central one. Benzodiazepine binding sites have also been described in thoracic muscles of the housefly (Abalis, Eldefrawi and Eldefrawi, 1983) as well as in the insect central nervous system (Lummis and Sattelle, 1985).

Flunitrazepam increased GABA responses in cultured cockroach brain neurones (Shimahara et al., 1987) via an increase in the frequency of channel opening as determined from noise analysis techniques. Flunitrazepam (1-10 $\mu$ M) reversibly increased GABA responses in thoracic ganglia of *Locusta migratoria* and *Schistocerca gregaria* (Lees et al., 1987). These workers also observed a reversible increase in the GABA response with 100 $\mu$ M pentobarbitone. Scott and Duce (1987) reported a similar enhancing action of pentobarbitone on the GABA response in locust muscle fibres. Another similarity to the vertebrate GABA<sub>A</sub> receptor lies in the possession of a TBPS binding site (Samaraj, Miller and Olsen, 1986; Eldefrawi and Eldefrawi, 1987).

In summary, insect brain and ganglia have a similar GABA receptor to vertebrates in that it operates a chloride channel, is activated by the same agonists (not baclofen) and is potentiated by benzodiazepines and barbiturates, but not inhibited by bicuculline. It remains to be seen whether the invertebrate benzodiazepine site can also be modulated by inverse agonists and receptor antagonists (Ro15-1788) like that observed in central mammalian benzodiazepine receptors.

#### 1.10 STEROID MODULATION OF THE MAMMALIAN GABA<sub>A</sub> RECEPTOR

Steroid hormones can act on the central nervous system to produce various neuroendocrine and behavioural effects. Most of the adrenal and gonadal steroids interact with intracellular receptors in the central nervous system and trigger genomically directed alterations in protein synthesis which occur in minutes to hours (Gorski, 1986). In addition to this intracellular long-lasting action, many steroids also produce more rapid effects on central nervous system excitability.

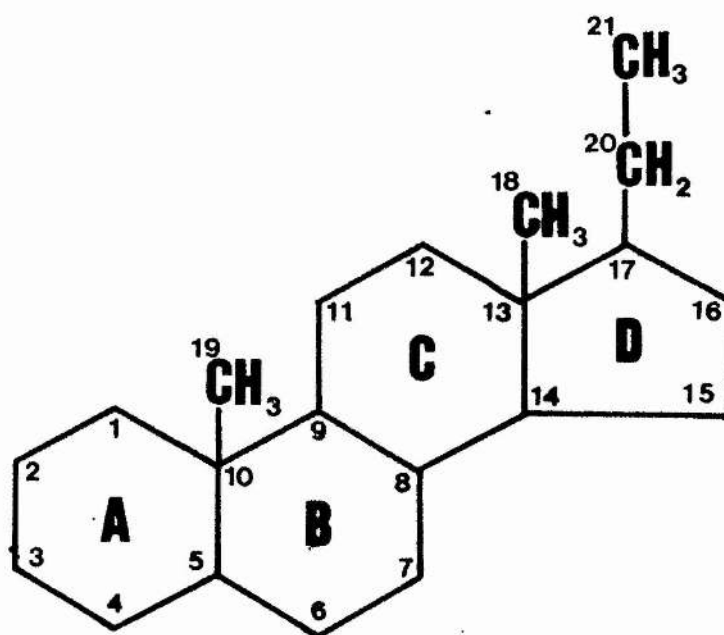
Indeed, Selye in 1941 described the rapid and reversible central nervous system depressant effects of various steroids in the rat. These steroids included progesterone and the mineralocorticoid deoxycorticosterone, as well as several of their metabolites. Some of the metabolites, were the most potent in inducing sedation and anaesthesia. Following these observations, synthetic steroids were developed to be used clinically as anaesthetics. One such steroid was 3 $\alpha$ -hydroxy-5 $\alpha$ -pregnane, 11, 20 dione (alphaxalone) (Childs, Currie, Davis, Dodds, Pearce and Wissell, 1971). The general structure of a steroid molecule and the nomenclature associated with it is shown in figure 1.0.

Since the anaesthetic actions of these steroids were rapid (seconds to minutes) an intracellular receptor mediated effect was unlikely. Some of these anaesthetic/sedative steroids may have an action on the GABA<sub>A</sub> receptor in the central nervous system analogous to other barbiturate anaesthetics such as pentobarbitone. The first relevant electrophysiological observation on the possible mechanism of action of alphaxalone was made by Scholfield in 1980. He observed a

Figure 1.0

An example of the structure of a steroid nucleus is shown opposite.

Numbers below 18 refer to C atoms within the rings; while 18-21 refer to C atoms in side chains. A-D designate the four rings in the steroid nucleus.  $\alpha$ -substituents project below the plane of the rings while  $\beta$ -substituents project above the rings.  $\alpha$ -substituents are represented by dotted lines whereas  $\beta$  substituents are represented by solid lines.



potentiation in the inhibition with the anaesthetic alphaxalone using intracellular recording in the neurones of the guinea pig olfactory cortex. At high concentrations of alphaxalone ( $>10\mu\text{M}$ ), a reduction in the membrane resistance was noted, along with a depolarization of up to 6mV, indicating a direct action on the membrane.

Similar reports were made by Harrison and Simmonds (1984) using extracellular recording in the rat cuneate nucleus, these workers described a potentiation of the GABA responses with alphaxalone (100nM-1 $\mu\text{M}$ ). The  $\beta$ -hydroxy isomer, betaxalone which is clinically inactive, however, had no effect (up to 30 $\mu\text{M}$ ). The potentiation observed with alphaxalone did not extend to glycine responses and was insensitive to Ro15-1788, which eliminated an action at the benzodiazepine site.

The interaction of alphaxalone with the GABA<sub>A</sub> receptor of bovine chromaffin cells was investigated using patch clamp techniques (Cottrell, Lambert, and Peters, 1986). Using the whole-cell voltage clamp technique, these authors observed potentiation of GABA-evoked currents with low doses of alphaxalone. At higher doses ( $>1\mu\text{M}$ ) alphaxalone directly evoked a membrane current which reversed at the chloride equilibrium potential. The alphaxalone evoked currents were reversibly suppressed by bicuculline. The latter results, suggested a direct agonist action of alphaxalone on the GABA<sub>A</sub> receptor at high concentrations.



At around the same period, Majewska, Bisslerbe and Eskay (1985) demonstrated an interaction of endogenous glucocorticoids (corticosterone and pregnenolone-sulphate) with the neuronal GABA<sub>A</sub> receptor. In ligand binding studies, using rat synaptosomal membranes and brain sections these workers described an enhancement in muscimol binding. This enhancement occurred via an increase in the affinity (K<sub>d</sub>) for the GABA site and was demonstrated with concentrations of steroids in the low nanomolar range. Electrophysiological characterization of this interaction soon followed using rat central neurones (Barker, Harrison, Lange, Majewska and Owen, 1986). These workers reported a potentiation of the GABA-evoked currents by alphaxalone and a progesterone metabolite 3 $\alpha$ -hydroxy-5 $\alpha$ -pregnane-20-one (100nM-1 $\mu$ M). Application of noise analysis techniques showed that the mean open time of the GABA gated channels shifted from 30ms to 74ms in the presence of 1 $\mu$ M alphaxalone. This prolongation of the open time was very similar to that caused by pentobarbitone (Study and Barker, 1981).

Other workers have also recently described potentiation of GABA action by alphaxalone in various central nervous system preparations. Harrison, Vicini and Barker, in 1987 showed that alphaxalone at low doses prolonged (5-8 fold) inhibitory postsynaptic currents in cultured rat hippocampal neurones. Potentiation of GABA action by alphaxalone has also been reported by Barker, Harrison, Lange and Owen (1987b) in cultured rat spinal neurones. Apart from the enhancing action on GABA responses, a direct membrane chloride conductance was also evoked at high ( $\mu$ M) concentrations. As shown in previous studies, glycine responses were unaffected by the steroid and

Ro15-1788 was ineffective in preventing the enhancement.

Alphaxalone also prolonged the duration of the cat dorsal root potential which is thought to be mediated in part by GABA and which is associated with presynaptic inhibition of excitatory transmission from primary afferents to motoneurons (Lodge and Anis, 1984). The direct agonist action of alphaxalone has also been described on the GABA<sub>A</sub> receptor of DRG neurones (Robertson, 1986).

Two metabolites of the steroid hormones progesterone and deoxycorticosterone 3 $\alpha$ -hydroxy-5 $\alpha$ -dihydroprogesterone (3 $\alpha$ -OH DHP) and 3 $\alpha$ -5 $\alpha$  tetrahydrodeoxycorticosterone (3 $\alpha$ -THDOC) respectively have been shown to interact with GABA<sub>A</sub> receptors in both binding and electrophysiological studies (Majewska, Harrison, Schwartz, Barker, and Paul, 1986). Both of these steroids inhibited the binding of TBPS and increased the binding of the benzodiazepine flunitrazepam to the GABA<sub>A</sub> receptor. In electrophysiological studies, these steroids enhanced the action of GABA in cultured hippocampal and spinal neurones.

The actions of two other progesterone metabolites 5 $\beta$ -pregnane-3 $\alpha$ -ol-20-one (pregnanolone) and 5 $\beta$ -pregnane-3-20-dione (pregnanedione) were investigated on the GABA<sub>A</sub> receptor of the bovine chromaffin cell (Callachan, Cottrell, Hather, Lambert, Nooney and Peters, 1986). Their effects were very similar to alphaxalone in that they potentiated GABA currents at low doses and at higher doses in some way directly activated the receptor. Pregnanedione (10 $\mu$ M) in outside-out patches induced single channel currents with conductances and reversal potentials similar to those activated by GABA, these

steroid evoked currents were also antagonized by bicuculline.

#### 1.10.1 OTHER ACTIONS OF STEROIDS ON MEMBRANE CURRENTS

The actions of the steroids on the GABA<sub>A</sub> receptor are quite specific, chloride currents activated by glycine are not enhanced by the steroids (Harrison and Simmonds, 1984; Barker et al., 1986, 1987b). The anaesthetic actions of the steroids could be brought about by a decrease in excitatory transmission and/or an increase in inhibitory GABA transmission. Effects of alphaxalone on aspects of excitatory transmission have been observed but only at relatively high concentrations. Gillo and Lass (1984) showed that alphaxalone in the  $\mu\text{M}$  range appeared to block open acetylcholine-activated channels in cultured chick myoballs. A suppression of acetylcholine-evoked currents in bovine chromaffin cells was also reported by Cottrell, Lambert and Peters (1986, 1987b) with concentrations of alphaxalone between 3-100 $\mu\text{M}$  ( $\text{IC}_{50} = 20\mu\text{M}$ ). Voltage dependent sodium currents were only minimally affected by 100 $\mu\text{M}$  alphaxalone. Intravenous administration of progesterone apart from significantly enhancing GABA mediated inhibitory responses also to a lesser extent suppressed glutamate excitation in rat Purkinje neurones (Smith, Waterhouse, Chapin and Woodward, 1987). These authors suggest that the anxiolytic, anticonvulsant and anaesthetic effects of progesterone may be brought about by its interaction with these two neurotransmitter systems.

A direct action of the sex steroid  $17\beta$ -oestradiol on the postsynaptic membrane of neurones in the rat medial amygdala has been shown (Nabekura, Oomura, Minami, Mizuno and Fukado, 1986). Using intracellular recording, these workers described a brief hyperpolarization which was related to an increase in the potassium conductance by  $1\text{nM}$   $17\beta$ -oestradiol. This effect was resistant to protein synthesis inhibitors (actinomycin D) and elimination of synaptic input to these neurones. The medial-amygdala is an oestrogen sensitive target tissue that is involved in the integration of chemosensory information. This example, suggests that the medial-amygdala neurones may monitor oestradiol levels in the cerebrospinal fluid, and possibly send information about the stage of oestrous cycle to other brain areas.

#### 1.10.2 STEREOSELECTIVITY OF STEROID ACTION

The actions of the steroids on the  $\text{GABA}_A$  receptor shows a strict stereoselectivity. Firstly, betaxalone (the  $3\beta$ -hydroxy isomer of alphaxalone) does not potentiate GABA action or directly activate its receptor (Harrison and Simmonds, 1984; Cottrell et al., 1987b; Barker et al., 1987b). Endogenous steroids which have now been shown to enhance the action of GABA are also known to possess anaesthetic activity (Harrison, Majewska, Harrington and Barker, 1987). From these studies certain structural aspects of the steroids have been implicated in their activity. These include

- (1) A  $5\alpha$  or  $5\beta$  reduced pregnane
- (2) A hydroxyl at C3 in the  $\alpha$  position

(3) A ketone (C=O) group at C20

Similar correlations between the inhibitory activity of steroids on the GABA<sub>A</sub> receptor and their anticonvulsant/ anaesthetic actions have been made (Gee, Chung, Chany, Brinton and McEwan, 1987). These workers using binding studies showed that steroids (nM range) inhibited the binding of TBPS to the GABA<sub>A</sub> receptor and that this interaction was not competitive. From the strict structural requirement of this interaction they proposed the existence of a distinct membrane bound "steroid site" which is coupled to the GABA<sub>A</sub> receptor complex.

The stereoselectivity of steroidal modulation of the GABA<sub>A</sub> receptor has been investigated using a combination of electrophysiological and radioligand binding techniques (Kirkness, Lambert, Peters, and Turner, 1987). Patch clamp techniques showed that 3 $\beta$ -hydroxy isomers of pregnane steroids lacked the ability to potentiate GABA currents in bovine chromaffin cells. This was confirmed in binding studies where these isomers only minimally enhanced [<sup>3</sup>H] muscimol binding in synaptosomal membranes from pig cortex. The 3 $\alpha$ -ol isomers, on the other hand potentiated GABA currents and also stimulated [<sup>3</sup>H] muscimol binding.

### 1.10.3 MECHANISM OF ACTION OF THE STEROIDS

Various different possibilities exist for the mechanism of action of steroids on the GABA<sub>A</sub> receptor, these include

- (a) Owing to their high lipid solubility, they may perturb the general membrane structure around the GABA<sub>A</sub> receptor.
- (b) They could perhaps bind to a hydrophobic region within the protein.
- (c) They could in some way bind to a protein/lipid interface within the membrane.
- (d) A distinct extracellular membrane site may exist where steroids can bind.

The potentiation of GABA action by steroids might occur from a physical effect of steroids dissolved in the neuronal membrane. Some evidence to support this notion comes from Makriyannis and Fesik (1983). These authors showed that alphaxalone perturbed artificial lipid membranes a property that was not shared by  $\Delta^{16}$ -alphaxalone (a double bond at C-16) even though the two steroids were incorporated equally into the membrane preparations.  $\Delta^{16}$ -Alphaxalone lacks anaesthetic activity and is 20-30 times less potent than alphaxalone at potentiating muscimol responses in the rat cuneate nucleus (Harrison, 1985). The actions of the steroids and the barbiturate pentobarbitone are very similar, in that they both prolong the GABA-evoked ipscs with little or any effect on their amplitude (Segal and Barker, 1984; Gage and Robertson, 1985; Harrison et al., 1987a). These observations indicate that the primary action of these compounds is to stabilize the GABA receptor channel complex in the open channel conformation.

Noise analysis techniques support this suggestion (Barker et al., 1986, 1987b; Study and Barker, 1981) where the mean open time of GABA-activated chloride channels is increased by these compounds. Single channel studies in excised outside-out patches provide more detailed information on the mechanism of action of the steroids. Callachan, Cottrell, Hather, Lambert, Nooney and Peters (1987a) showed no action of pregnanolone and pregnanedione on the amplitude of GABA-activated chloride channels in outside-out patches from chromaffin cells. However these steroids greatly prolonged the burst duration of the single channel currents.

The lack of effect of Ro15-1788 on the enhancement of GABA action by steroids rules out a possibility of an interaction at the central benzodiazepine receptor. Although the actions of the steroids are very similar to the barbiturate pentobarbitone, it is unlikely that the steroids act at the same site since the steroid induced currents were greatly potentiated by phenobarbitone (Cottrell et al., 1987b; Callachan et al., 1987a). Further evidence for separate sites came from experiments, that showed that 1mM pentobarbitone evoked whole-cell currents were enhanced upon exposure to 500nM pregnanolone in bovine chromaffin cells. Also unitary currents evoked by pregnanolone were potentiated by 500µM phenobarbitone (Callachan, Lambert and Peters, 1987b).



#### 1.10.4 PHYSIOLOGICAL SIGNIFICANCE OF STEROID ACTION

Both  $3\alpha$ -OH DHP and  $3\alpha$ -THDOC are present in the brain, as are their biosynthetic enzymes  $5\alpha$ -steroid reductase and  $3\alpha$ -hydroxy steroid oxidoreductase (Karavolas, Betrics, Hodges and Rudie, 1984). Also THDOC, a major metabolite of deoxycorticosterone is released in elevated concentrations from the adrenal cortex after rises in adrenocorticotrophic hormone such as those that occur during exposure to stressful stimuli (Schambelon and Biglieri, 1972). The anxiolytic action of THDOC is confirmed in the Vogel (rat punished drinking) test (Crawley, Glowa, Majewska and Paul, 1986).

The progesterone metabolite  $3\alpha$ -hydroxy- $5\alpha$ -pregnane-20 one has been shown to possess analgesic action (Kavaliers and Wiebe, 1987). Intracerebroventricular administration of this steroid increased the latency in a heat foot shock test in rats. The analgesic action was prevented by peripheral administration of the GABA antagonists bicuculline and picrotoxin. The  $3\beta$ -hydroxy isomer, however, failed to affect the nociceptive response. All of the actions of the steroids described so far have GABA enhancing actions, however, there may be endogenous steroids which can decrease the action of GABA. One such steroid is pregnenolone-sulfate (Majewska and Schwartz, 1987), this steroid competitively inhibited TBPS binding and antagonized pentobarbitone-stimulated [ $^3$ H] flunitrazepam binding to synaptosomes. In addition pregnenolone-sulfate inhibited muscimol-stimulated [ $^{36}$ Cl $^-$ ] uptake in brain synaptosomes indicating an antagonistic action on the GABA $_A$  receptor. This steroid has yet to demonstrate its antagonist action in electrophysiological studies.

Since the GABA<sub>A</sub> receptor complex appears to be bimodally regulated by various endogenous steroids, it is possible that an imbalance between excitatory and inhibitory steroids in plasma or the central nervous system (determined by the activity of steroidogenic tissues) could lead to hypo- or hyper-activity of central GABA<sub>A</sub> receptors with important pathophysiological consequences.

Barnes (1986) has suggested that steroids may influence changes in mood by slight changes in the H.P.A. (hypothalamus-pituitary-adrenal) axis. Normally the hypothalamus releases corticotropin releasing hormone which signals the pituitary gland to release adrenocorticotrophic hormone (ACTH). ACTH then triggers the outer portion of each adrenal gland to release its steroid hormones. However, in Cushings disease, depressive illness and stress, the adrenals release greater than usual amounts of steroid hormones into the bloodstream. This may result in higher circulating levels of steroids that depress nerve cell function. It is possible that many of the mood changes associated with stress, the menstrual cycle and pregnancy may be related to the effects that steroids and their metabolites have on neurones in the brain. Also with depression, Cushings disease, or anorexia nervosa, when blood steroid levels increase, mood changes such as anxiety and depression may be even more pronounced.

### 1.11 GABA-ACTIVATED SINGLE CHANNEL CURRENTS IN VERTEBRATES

GABA has been shown to activate multiple conductance states in various mammalian cells. In cultured mouse spinal neurones, Bormann, Hamill and Sakmann (1987) have observed four distinct conductance states for the GABA receptor of 12, 19, 30 and 44pS and four similar conductance states of 12, 19, 30 and 46pS for the glycine receptor in outside-out patches with symmetrically distributed chloride solutions. The dominant GABA state was 30pS with the 12 and 44pS states being observed only very occasionally; the dominant glycine state was 46pS with the 12 and 19pS state being activated only rarely. Distinct transitions between the different conductance states were observed (Hamill, Bormann and Sakmann, 1983).

Multiple conductance states have also been reported for the GABA<sub>A</sub> receptor of pituitary intermediate lobe cells (Taleb, Demeneix, Feltz, Bossu, Dupont and Feltz, 1987). These workers obtained conductances of 22, 31, and 46pS for the GABA<sub>A</sub> receptor using symmetrical chloride solutions. GABA also activated multiple conductances in bovine chromaffin cells of 11, 18, 31, 37 and 45pS (Cottrell et al., 1985; Bormann and Clapham, 1985). The number of states and the dominant conductance level activated by GABA varied from patch to patch (Bormann and Clapham, 1985). McBurney, Smith and Zorec (1985) observed 3 similar conductance states for the GABA<sub>A</sub> and glycine receptors of 20, 30 and 45pS in rat spinal neurones. Multiple conductance states have also been described for the GABA<sub>A</sub> receptor in outside-out patches from cultured chick cerebral neurones (Weiss, Barnes, and Hablitz, 1988). Conductances of 6-8pS, 10-13pS, 20pS, and 27-32pS were shown with the dominant level being the 20pS state.

Clear transitions between these different conductance levels were also reported.

Allen and Albuquerque (1987) using inside-out patches from cultured embryonic hippocampal neurones observed a single conductance state of approximately 19pS. A GABA-activated single channel conductance of 19pS was also found in cultured bovine lactotrophs (Inenaga and Mason, 1987). In all the above studies, the I-V relationships for the single channel currents activated by GABA were linear no evidence of rectification was reported.

There is, however, one description of rectification of GABA-activated chloride channels in adult hippocampal neurones from the guinea-pig (Gray and Johnston, 1985). These workers used proteolytic enzymes to enable gigaohm seals to be obtained on pyramidal and granule cell membranes. I-V relationships showed outward rectification with the channel having a conductance of 20pS at negative patch potentials and this value increased to almost 40pS at a patch potential of +40mV.

#### 1.12 OTHER AGENTS INTERACTING WITH THE GABA<sub>A</sub> RECEPTOR.

Various different agents have been shown to interact with the GABA<sub>A</sub> receptor. One such agent is penicillin which has been reported to cause convulsions *in vivo*. Chow and Mathers (1985), using patch clamp techniques in mouse spinal neurones showed that sodium benzylpenicillin (PCN) at concentrations greater than 0.2mM reversibly reduced the GABA response. PCN (2mM) reduced the mean open time of GABA-gated single channel currents in isolated outside-out patches.

The authors suggested that penicillin blocked open GABA-gated single channel currents and that this effect could possibly contribute to its convulsant action.

A recently introduced technique which is going to prove very useful in the future is the functional expression of brain neurotransmitter receptors from brain mRNA injected into *Xenopus laevis* oocytes (Constanti, Houamed, Smart, Bilbe, Brown and Barnard, 1984). The GABA<sub>A</sub> receptor expressed from brain mRNA is pharmacologically similar to the neuronal GABA<sub>A</sub> receptor in that bicuculline (competitively) and picrotoxin (non-competitively) antagonized the GABA response. Also, the benzodiazepine chlorazepate and pentobarbitone potentiated the GABA response (Scholfield, Darlison, Fujita, Burt, Stephenson, Rodriguez, Rhee, Ramachandran, Reale, Glencourse, Seeburg and Barnard, 1987).

Voltage clamp techniques applied to the *Xenopus laevis* oocyte showed that TBPS reversibly inhibited the GABA evoked current in a dose dependent manner. TBPS (25-100nM) showed a mixed type of antagonism which was neither competitive nor a simple pure channel block (Van Renterghem, Bilbe, Noss, Smart, Constanti, Brown and Barnard, 1987; Barnard, Bilbe, Houamed, Moss, Van Renterghem, and Smart, 1987). These authors suggest that TBPS perhaps stabilizes a closed conformation of the liganded-receptor channel complex. Just recently a new set of GABA antagonists have been synthesized by Wermuth and Biziere (1986). These antagonists are pyridazinyl GABA derivatives and have been called SR95103, SR95531 and SR42641. SR42641, in electrophysiological tests has been found to selectively block GABA<sub>A</sub> responses in rat DRG neurones. Dose response curves were

shifted to the right in a parallel fashion indicating competitive interaction similar to that of bicuculline. GABA<sub>B</sub> interactions with the calcium current were unaffected by SR42641 (Desarmenien et al., 1987).

Various divalent cations have been reported to affect mammalian GABA<sub>A</sub> responses. Intracellular calcium ions were first shown to affect GABA<sub>A</sub> responses in sensory neurones from the bullfrog *Rana-cutesbiana* in 1986 (Inoue, Oomura, Yakushiji, and Akaike, 1986). These workers showed that an increase in internal calcium ions suppressed the GABA-activated chloride conductance in these neurones. Using this preparation, these workers investigated the interaction further, they found that a preceding calcium current greatly suppressed the GABA current and that the GABA dose-response curve was shifted to the right without changing the maximum response by increasing the internal calcium ions (Inoue, Tukutomi and Akaike, 1987; Inoue, Sadoshimi and Akaike, 1987). These observations have also been confirmed in inside-out patches from the sensory neurones where  $1\mu\text{M} [\text{Ca}^{2+}]_i$  decreased the open probability of GABA-activated single channels compared with  $0.01\mu\text{M} [\text{Ca}^{2+}]_i$  (Akaike, Ikemoto, Kanedo, and Ono, 1987). These workers have suggested the existence of a calcium receptive site in the membrane interior which is in some way coupled to the GABA<sub>A</sub> receptor.

Kaneko and Tachibana (1986) using the whole-cell patch clamp technique described blocking effects of cobalt and related ions on the GABA-evoked chloride current in turtle retinal cones. Cobalt, nickel, and cadmium in the  $\mu\text{M}$  range blocked the GABA-evoked currents non-competitively.  $5\mu\text{M}$  cobalt reduced the GABA current by 50% and the



block was not voltage dependent over the physiological range of potentials employed. Micromolar concentrations of zinc have also been shown to antagonize the NMDA and GABA responses in cultured hippocampal neurones. Zinc was a non-competitive antagonist of NMDA responses, this antagonism was not voltage sensitive. Zinc had no effect on kainate or quisqualate responses. GABA responses were also reduced by approximately 55% by 5 $\mu$ M zinc (Westbrook and Mayer, 1987).

### 1.13 SUITABILITY OF DISSOCIATED NEURONES MAINTAINED IN CULTURE

Detailed study of the excitable membrane properties of individual neurones in the mammalian central nervous system is very difficult owing to the technical limitations in performing experiments in the intact central nervous system. Electrophysiological study of neurones dissected from the embryonic and adult central nervous system and maintained in dissociated cell culture have provided an excellent alternative to *in vivo* studies.

These cultured cells have many of the properties characteristic of neurones studied *in vivo* including chemical and electrical excitability and spontaneous changes in electrical activity. The neurones grow as a monolayer on top of glial and fibroblast cells and can be fairly readily identified on morphological grounds. The recently introduced patch electrode technique of Hamill, Marty, Neher, Sakmann and Sigworth (1981) is particularly suited to cultured cells since invariably their membranes are "clean" and do not require enzyme treatment to obtain gigaohm seals.

Binding studies of [ $^3\text{H}$ ] GABA to cultured mouse spinal neurones have been described (Ticku, Haung and Barker, 1980) as well as benzodiazepine (clonazepam and Ro5-4864) receptor binding (Sher, 1983). Numerous GABAergic synapses have been found immunocytochemically on the somato of spinal neurones (McLaughlin, Barber, Saito, Roberts and Wu, 1975). The morphological and intrinsic membrane properties of cultured mouse spinal neurones were first described in a series of papers by Ransom, Neale, Henkart, Bullock and Nelson in 1977. Since then several workers have used these neurones as a model to elucidate GABA function and its modulation in the central nervous system.

The rat DRG neurone has been shown to possess both GABA<sub>A</sub> and GABA<sub>B</sub> receptors (Desarmenien, Desaulles, Feltz and Hamann, 1987). The somato of DRG neurones despite having GABA<sub>A</sub> receptors apparently lack any GABAergic innervation (Gallagher and Gallagher, 1983). The physiological significance of this remains unknown but this receptor shares many of the features associated with the brain and spinal GABA<sub>A</sub> receptor, making it a useful system for studying the electrophysiological actions of GABA.



#### 1.14 AIMS AND OBJECTIVES

GABA is an important transmitter in the nervous system. GABA has been shown to interact via two different receptors, the GABA<sub>A</sub> and GABA<sub>B</sub> receptor. The former acts by increasing Cl<sup>-</sup> conductance, while the latter operates cation selective channels. Several clinically important drugs appear to interact with the GABA<sub>A</sub> receptor. It is therefore important to understand the actions of these drugs at the cellular and molecular levels.

Dissociated cultured neurones are a suitable preparation for the study of GABA action and have been used by several workers. In this work, cultured mouse spinal and rat DRG neurones were used to investigate the actions of GABA and compounds which affect the GABA<sub>A</sub> receptor. This was done by using both the whole-cell and single-channel recording configurations of the patch-clamp technique. The modulation of GABA action by diazepam, barbiturates and steroids was studied, however, during the course of the study spontaneous events were observed in isolated outside-out membrane patches from both types of neurones. These spontaneous currents had several different conductance, some of which were similar to those activated by GABA. Owing to this similarity and their frequent occurrence, their nature and ionic dependence was studied in greater detail.

## METHODS



## METHODS

The experiments described in this thesis were performed on isolated mouse spinal neurones and neonate rat dorsal root ganglion (DRG) neurones maintained in culture. The visual resolution and the experimental inaccessibility of the brain and spinal cord, make cultured cells an attractive material for the study of the pharmacology of individual cells.

### 2.1 PREPARATION AND DISSOCIATION OF MOUSE SPINAL NEURONES

Spinal cord neurones from the foetal mouse were maintained in culture as described previously by Ransom, Neale, Henkart, Bullock and Nelson in 1977. Spinal cords were removed from 12-14 day old mouse C57BL/10 embryos (gestation term = 21 days). The age of the foetus, was critical in that younger or older ones did not produce neurones suitable for patch-clamp recording.

The pregnant mice were killed by placing them in a CO<sub>2</sub> atmosphere for 2-3 minutes. Their necks were fractured, when respiration had ceased. The mice were then placed on a sterile stainless steel tray and flooded with 70% ethanol. With large scissors a cut was made in the skin. The skin was prised apart with the fingers, revealing the digestive system and the embryos beneath the peritoneum.

Using fine scissors, the peritoneum was cut and the embryos carefully lifted out of the mouse. Ethanol at 70% was squirted onto the embryonic sacs to remove any excess blood. Litter size varied from mouse to mouse but was usually in the range 4-11, the average being approximately 7-8.

The embryonic sacs were placed along the edge of the tray and longitudinal cuts made in the sacs. The embryos were rolled out into an adjacent dish containing 2ml of D<sub>1</sub>SGH (dissecting medium containing 10 Hanks salt solution with glucose/sucrose and Hepes buffer) solution.

When all the embryos had been collected, they were transferred to another culture plate containing 2ml of D<sub>1</sub>SGH to remove any remaining blood from the embryos. Under a dissecting microscope, a cut was made just below the head and a cut down the spinal cord was made with fine dissection scissors. The spinal cord was carefully rolled out with the attached dorsal root ganglia into another culture plate containing 1ml of D<sub>1</sub>SGH. Once all the cords were removed, they were transferred to a laminar flow cabinet, where the D<sub>1</sub>SGH solution was carefully sucked out with a Pasteur pipette.

1.0ml of a 0.25% trypsin solution was added to the cords and the covered plate placed in a 90% air/10% CO<sub>2</sub> Forma incubator at 37°C for 30 minutes. After the elapsed time the plate was removed and the action of trypsin arrested, with the addition of 1ml of 10/10 medium (minimum essential medium (MEM) with 10% donor horse serum and 10% foetal calf serum).

The contents of the plate were transferred to a test tube. The spinal cords were now triturated (mechanically dissociated). A Pasteur pipette was used to take up and gently expel the cords 10 times; care being taken not to introduce any air bubbles. The trituration protocol was repeated several times with consecutively smaller diameter Pasteur pipettes, this was repeated until all the cells were in suspension. A further 8ml of 10/10 medium was added to the test tube and the resulting suspension mixed thoroughly. A 0.5ml sample was removed and the cells counted on a Coulter Counter.

Neurones were plated out at a density of 120,000 per 35mm collagen coated plate. An aliquot (0.5-1.0ml according to the count obtained) of the 9.5ml cell suspension was added to each collagen coated culture plate and the total volume made up to 1.5ml with 10/10 medium.

The tray containing the plates was shaken gently from side to side and to and fro in order to obtain an even distribution of neurones. The freshly plated cultures were then kept in the 90% air/5% CO<sub>2</sub> atmosphere. On the odd occasion pure spinal cord cultures without the attached ganglia were produced. However no significant differences in the electrophysiological properties were observed with these cultures and those with DRG neurones. It was often possible to do two dissections on the one day, this proved necessary to maintain stocks as periodically cultures would become infected (bacteria, yeast or fungi) and had to be discarded. Since no antibiotics were used in the medium, sterile conditions were used during the dissection and

careful monitoring of the cultures proved necessary.

## 2.2 COLLAGEN COATING OF CULTURE PLATES

Collagen coated culture plates were usually prepared the day prior to the dissection. A collagen solution was made by adding 50mg of calf-skin collagen to 100ml of sterile 1:1000 glacial acetic acid solution. This mixture was stirred at room temperature for up to 12 hours. One drop of the collagen solution from a Pasteur pipette was sufficient to spread and coat a 35mm culture plate. The plates were covered and left to dry in the laminar flow cabinet overnight.

## 2.3 MAINTENANCE OF CULTURES

The cultures were kept for 3-5 days before their first change with 1.5ml of 10/10 medium. After a further 1-3 days, a second change was performed, usually by this time the non-neuronal (glial, fibroblasts) cells were usually confluent. This change was done using medium from which foetal calf serum was omitted (ie 10 medium) and to which was added 5 fluoro-2- deoxyuridine (FuDR) at 15ug/ml. The FuDR served to control the rapid division of the non-neuronal cells. If the non-neuronal cells were not confluent, this change with FuDR was postponed until the next change 2 days later. After 2-3 days in FuDR the medium was completely replaced with 10 medium alone. The cultures were then changed 2-3 times a week with 10 medium.

## 2.4 DEVELOPMENT AND MORPHOLOGY OF NEURONES

Soon after plating, the cells were round and in small clumps such that it was difficult to distinguish neurones from non-neuronal cells. However, after 24 hours flat background (non-neuronal) cells had spread over the collagen surface, and small neurones 5-10 $\mu$ m in diameter with thin processes up to 50 $\mu$ m in length could be observed alone or extending from clumps. Figure 2.1a shows a photograph of spinal neurones after 1 day in culture. For the first 2-3 weeks in culture spinal neurones displayed an increase in the diameter of their soma, as well as extent of process outgrowth and complexity. An example of a typical spinal neurone after 14 days in culture is shown in figure 2.1b. During the second and third week some loss of neurones occurred after which the neurones normally stabilized in terms of their number and size. Cultures could occasionally be kept for up to 2 months.

Mouse DRG and spinal neurones could be identified on morphological and electrophysiological grounds. DRG neurones were characterized by large round phase bright cell bodies, very few (one or two) small diameter processes and a sharply defined nuclei and nucleoli.

Spinal cord neurones are generally smaller, have long tapering processes which form complicated branching patterns and the neuronal nuclei are usually less well defined. In a few of the cultures some spinal neurones were found in clumps and had round cell bodies and few if any processes.

Figure 2.1a

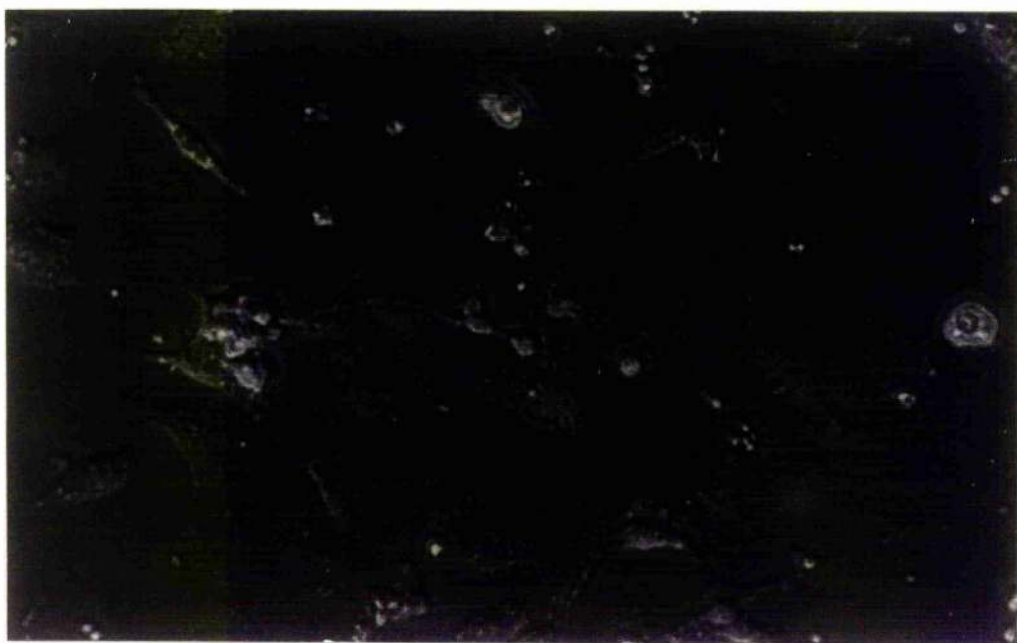
A photograph showing the appearance of spinal neurones after 1 day in culture. Note the small diameter processes extending from the soma.

Figure 2.1b

A photograph showing the appearance of a spinal neurone after 14 days in culture. The photograph shows the several complex tapering processes extending from the soma which is a characteristic trait of spinal neurones.



a



$\overline{30\mu\text{m}}$

b



$\overline{30\mu\text{m}}$

Further categorization of the cell type could be made using electrophysiological techniques. Spinal neurones often exhibited synaptic currents under whole-cell voltage clamp conditions. In current clamp mode, virtually all spinal neurones showed spontaneous synaptic potentials and action potentials (see Results). DRG neurones showed no spontaneous synaptic potentials or action potentials, however they could be made to fire action potentials upon the injection of depolarizing current into the cell (see Results). With the above combination of morphological and electrophysiological criteria, the cell type could be unambiguously identified. Patch clamp recordings were performed on spinal neurones that were maintained in culture for between 6-60 days and small (10-20 $\mu$ m) neurones were preferentially selected.

## 2.5 PREPARATION OF RAT DRG NEURONES

Dorsal root ganglion (DRG) neurones were isolated and maintained in culture as described in 1985 by Forda and Kelly. Briefly, 1-3 day old rats were killed by placing them in a jar containing ether for 2-5 minutes. The spinal column was then removed from the rat.

The ganglia were carefully removed from the column and placed into a culture plate containing 1ml of Dulbeccos PBS (phosphate buffered saline-calcium and magnesium free) solution. The plate was then placed in a 95% air/5% CO<sub>2</sub> incubator with a 0.25% trypsin solution for 25-30 minutes. The trypsin solution was transferred to a centrifuge tube and centrifuged at 20g for 1 minute.

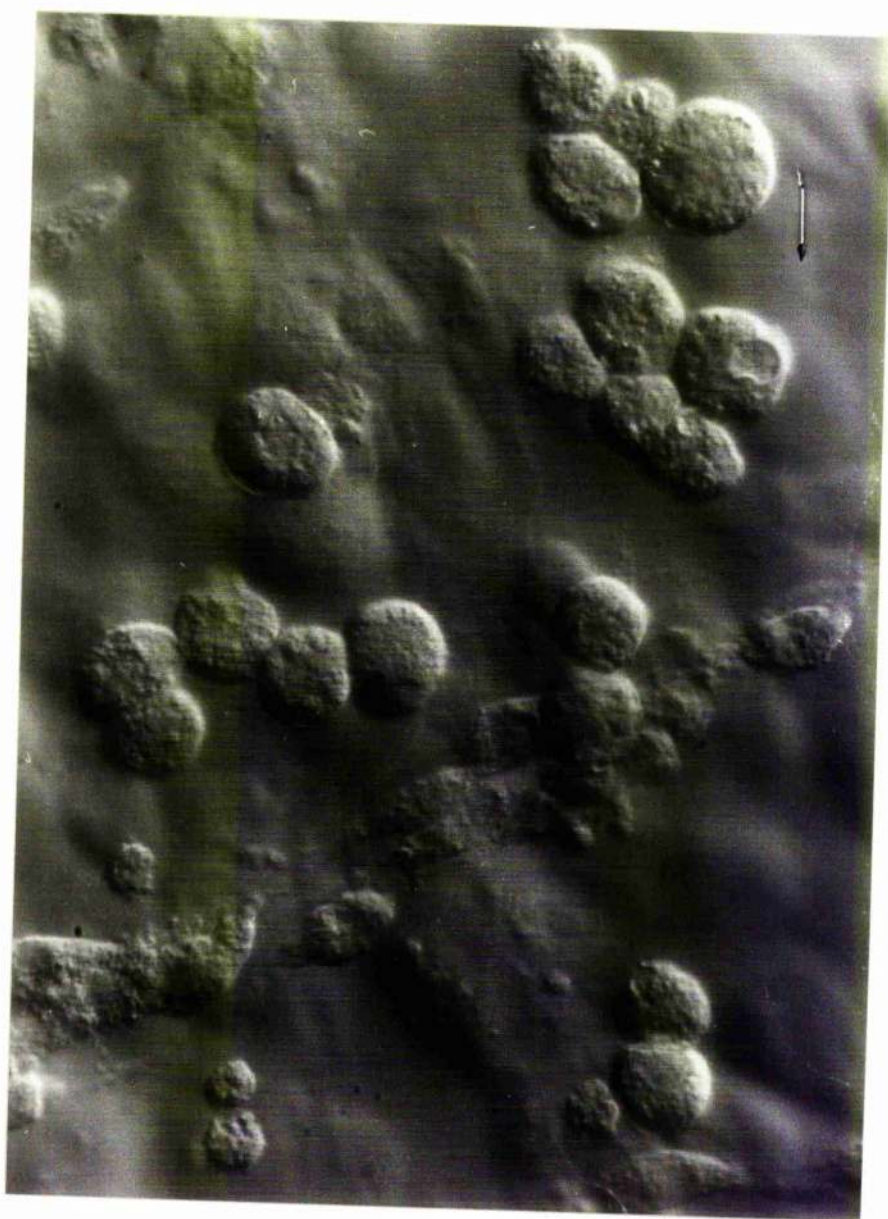
The supernatant (trypsin containing solution) was discarded. The ganglia were centrifuged again in a 1ml DNase (40µg/ml deoxyribonuclease in PBS) solution at 100g for 5 minutes. The supernatant again discarded, and a further 1ml of DNase added. A Pasteur pipette was used to triturate the ganglia 5 times, and the resulting suspension recentrifuged at 100g for 5 minutes. The supernatant was discarded and 1ml of 10 medium (Minimum Essential Medium (GIBCO) containing 10% foetal calf serum) added. Further trituration with successively smaller diameter Pasteur pipettes was performed. This procedure was repeated until a cell suspension had been formed.

The cell suspension was added to the centre of each culture plate containing approximately 1.5ml of 10 medium. This centreing proved necessary to provide adequate access for the patch and pressure pipettes. From each rat approximately 2 plates of cells could be obtained. The covered plates were then kept in a 95% air/5% CO<sub>2</sub> incubator until required. The 10 medium used was supplemented with 4mM glutamine, antibiotics (penicillin/streptomycin each at 50µg/ml) and nerve growth factor (NGF at 1µg/ml).

Patch clamp recordings were made from DRG neurones maintained in culture from between 1-10 days. DRG neurones obtained from the rat were similar in appearance to those obtained from the foetal mouse, in that they had round phase bright cell bodies, few processes if any and sharply defined nuclei and nucleoli. Figure 2.2 shows a photograph of DRG neurones maintained in culture for 2 days. Their size were normally between 10-30µm in diameter.

Figure 2.2

A photograph showing the appearance of rat DRG neurones after 2 days in culture using Nomarski optics.



$\overline{10\mu\text{m}}$

## 2.6 PATCH CLAMP METHODS

Patch-clamp recording was made possible by the discovery that under certain circumstances the membrane of a cell can seal extraordinarily tightly to the tip of a glass pipette. Patch clamp recording was first performed on denervated frog muscle fibres (Neher and Sakmann, 1976). Single-channel currents were obtained using different cholinergic agonists in the patch pipette using the cell-attached patch configuration. In order to achieve high resistance seals, the cell surface had to be treated with enzymes to leave a clean membrane surface.

The next major advancement came with the introduction of cultured cells. Gigaohm ( $10^9 \Omega$ ) seals in the range 10-100G $\Omega$  were fairly readily obtained on cultured cells, these gigaohm seals reduced the background noise further and substantial voltage differences could be imposed across the membrane without causing large noisy leakage currents. The most surprising aspect of the gigaseal was that it was mechanically very strong.

Withdrawing the pipette from the cell, the seal still remains intact and a portion of membrane is pulled off the cell. If this withdrawal occurs in a low  $\text{Ca}^{2+}$  solution, an inside-out cell free membrane patch is formed at the tip of the pipette, whose internal face is exposed to the bathing solution. In the cell-attached patch mode, if further suction is applied to the pipette, the membrane patch can be ruptured, thereby producing a low resistance access to the cell interior. This is the whole-cell clamp configuration which is applicable to small cells. Once the whole-cell clamp configuration is

of the pipette results in the formation of a functional membrane patch over the tip of the pipette. This membrane patch has its external face oriented towards the bathing solution. This is the outside-out patch. By 1981 the gigaseal technique, the instrumentation and the various recording configurations had been described in detail by Hamill, Marty, Neher, Sakmann and Sigworth. Figure 2.3 shows a schematic representation of the different configurations of the patch-clamp technique.

## 2.7 GLASS CAPILLARIES FOR PIPETTES

The patch clamp experiments described in this thesis were performed using hard borosilicate glass (Clark Electromedical GC150T-15) having external and internal diameters of 1.50 and 1.05 mm respectively. The main advantages of using hard (thick) walled glass was that they had better noise characteristics than soft glass. Also thick walled glass tends to have the greater success rate of obtaining gigaseals and these tended to be more stable than those obtained with soft glass (Corey and Stevens, 1983).

The 15cm borosilicate glass was cut in half and the ends were rounded by briefly placing them over a hot bunsen flame. This proved necessary to prevent scraping of the chloride from the silver chloride wire when inserting the pipette into the pipette holder.



Figure 2.3

A schematic representation of the different configurations of the patch clamp technique (modified after Hamill et al., 1981).

a The formation of a low resistance ( $M\Omega$ ) seal upon contact of the pipette with the membrane.

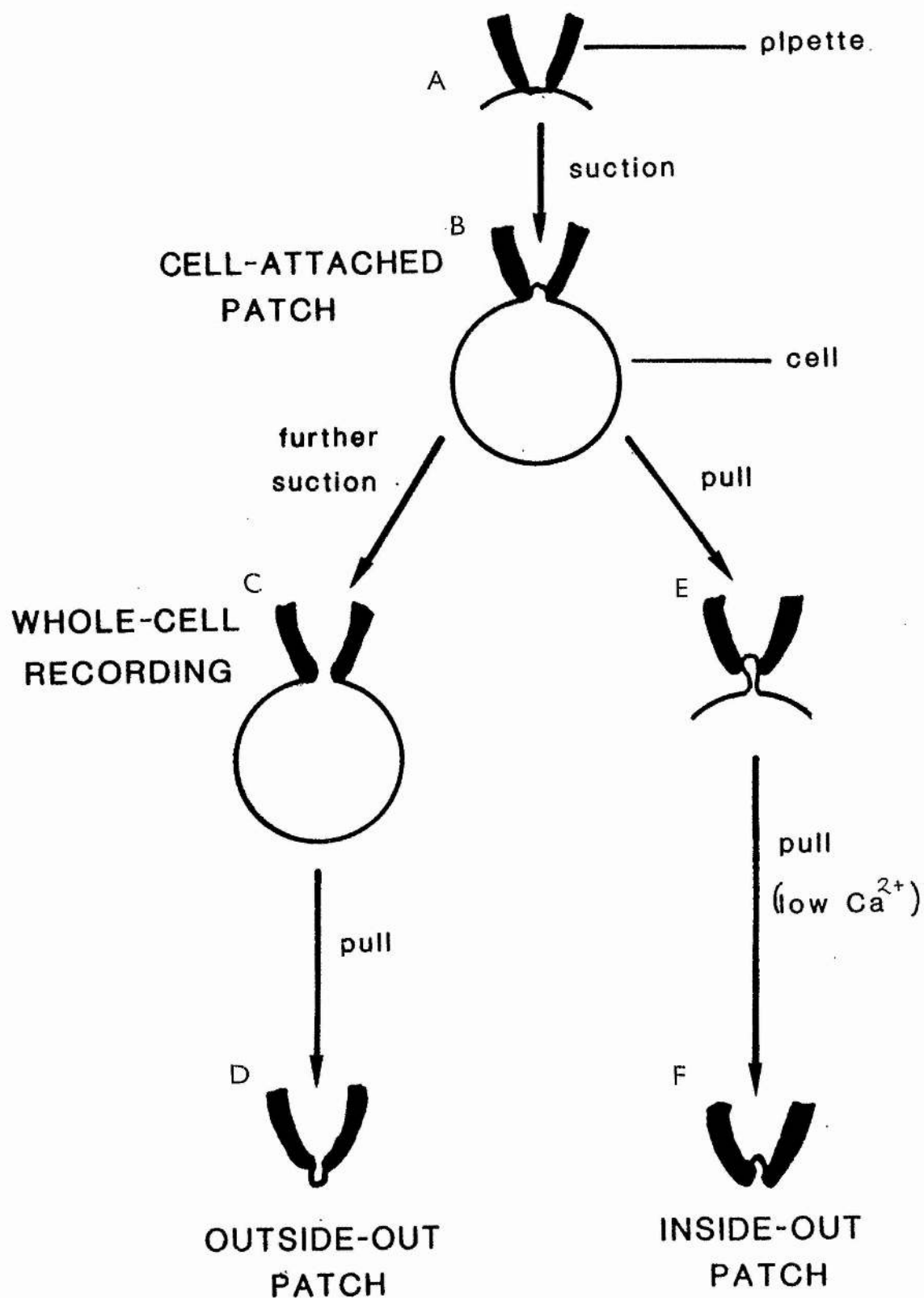
b A high resistance seal ( $>10G\Omega$ ) formed by gentle suction applied to the pipette; this is the cell-attached patch configuration.

c Further suction disrupts the membrane spanning the pipette this is the whole cell recording configuration.

d Slow withdrawal of the pipette results in resealing of the membrane to the pipette causing the formation of the outside-out patch.

e & f After the formation of a cell-attached patch pulling the pipette away from the cell (e) can result eventually in the formation of an inside-out patch (f) when the cell is bathed in a low  $Ca^{2+}$  solution.





## 2.8 PULLING PIPETTES

Patch pipettes were pulled in a two stage process using a vertical patch pipette puller (Narishige PP83). The first stage pulled the glass to 200-400 $\mu$ m over a narrow region of 7-10mm. The heating filament was recentred over the constriction and a second pull performed, which thinned the glass further to the point at which two separate pipettes were formed.

Both symmetrical pipettes were used in experiments. The length of the first pull and the temperature of the second pull were the main factors in determining the final tip diameter of the pipette. Pipettes at this stage had resistances of 1-2M $\Omega$  when filled with physiological solution. Generally pipettes have to be fire-polished in order to obtain gigaseals. A scanning electron micrograph of a pipette before polishing is shown in figure 2.4a.

## 2.9 COATING PIPETTES WITH SYLGARD

For some single-channel recording experiments patch pipettes were coated with an insulating agent Sylgard<sup>R</sup> (Dow Corning Corporation) to improve the signal to noise ratio. Sylgard<sup>R</sup> was precured by mixing the resin and catalyst oil in ratios of approximately 10:1. The Sylgard was applied as close to the pipette as possible with the naked eye using a bent syringe needle. The Sylgard was cured by placing the pipette in a hot coil for a few seconds.

In spite of applying Sylgard to patch pipettes, the major source of noise in single-channel recording experiments proved to be the amount of bath solution in the culture plate. To minimize this noise the amount of bath solution was decreased to the lowest level possible which was approximately 1.5-2.0ml.

## 2.10 HEAT POLISHING PIPETTES

Heat (fire) polishing was used to smooth the edges of the pipette tip and to remove any contaminants left on the tip from Sylgard coating. The pipette was placed on a modified microscope at a magnification of 375 times. A V shaped platinum-iridium filament provided the heat source.

An airstream directed at the filament created a steep thermal gradient which confined the polishing to the tip of the pipette. The tip of the pipette and the filament were brought into focus and the pipette slowly advanced towards the glowing filament. A visible constriction and darkening of the tip indicated polishing to have occurred. The extent of which was determined by the bubble test.

## 2.11 BUBBLE TEST

The pipette was connected to a 10ml syringe with tight fitting polyethylene tubing. The tip was immersed in methanol when pressure was applied, bubbles eventually emerged from the tip. This occurred when the pressure exceeded that produced by the surface tension of the air-methanol interface. Since the surface tension is inversely proportional to the radius of curvature, large diameter tips produced bubbles at lower pressures than small diameter tips.

The bubble test was used as a rough and convenient measure of the tip diameter. Heat polishing is normally a pre-requisite for gigaseal formation. A scanning electron micrograph of a polished patch pipette is shown in figure 2.4b. Tips of polished pipettes have a smooth appearance compared to that of unpolished pipettes shown in figure 2.4a. Polished patch pipettes were then kept covered in a Petri dish until required for experiments.

## 2.12 FILLING PATCH PIPETTES

To enable gigaohm seals to be obtained, the pipette solution and the tip have to be exceptionally clean. Therefore all patch pipette solutions were filtered through a 0.22 $\mu$ m pore size filter (Millipore cat. no. GSW PO 2500). The filtered solutions were placed in a small beaker. Patch pipettes were connected to a tight fitting syringe similar to that used for bubble testing. The pipette tip was immersed in the filtered solution and suction applied with the syringe, this resulted in a small volume of the solution being drawn

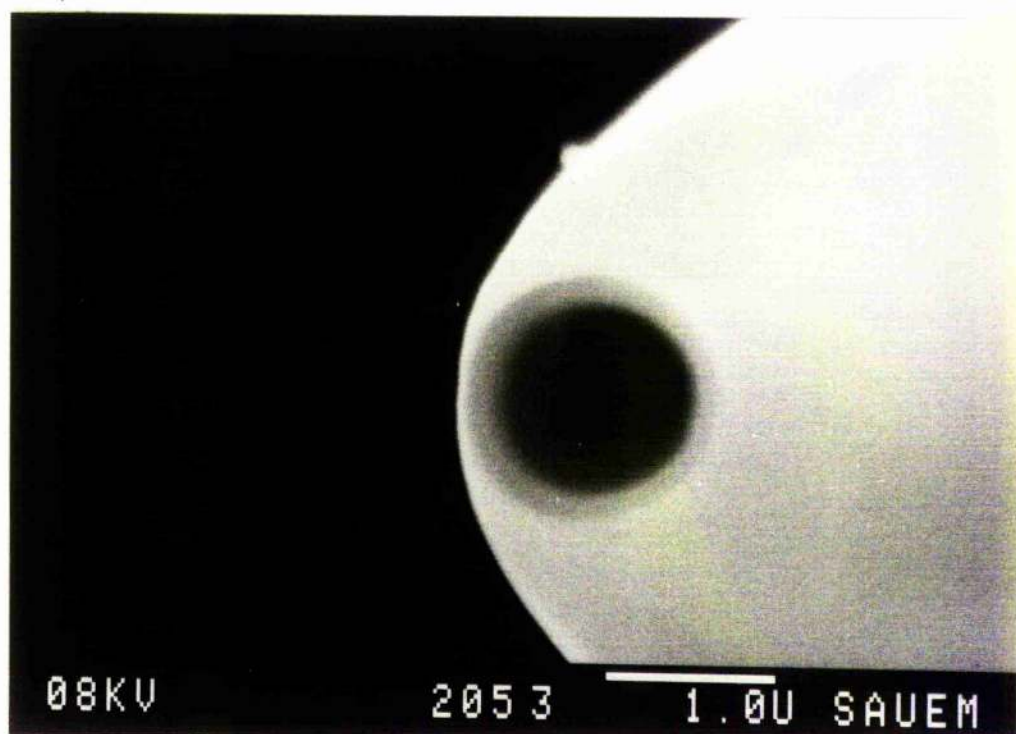
Figure 2.4a

A scanning electron micrograph of an unpolished patch pipette suitable for spinal neurones.

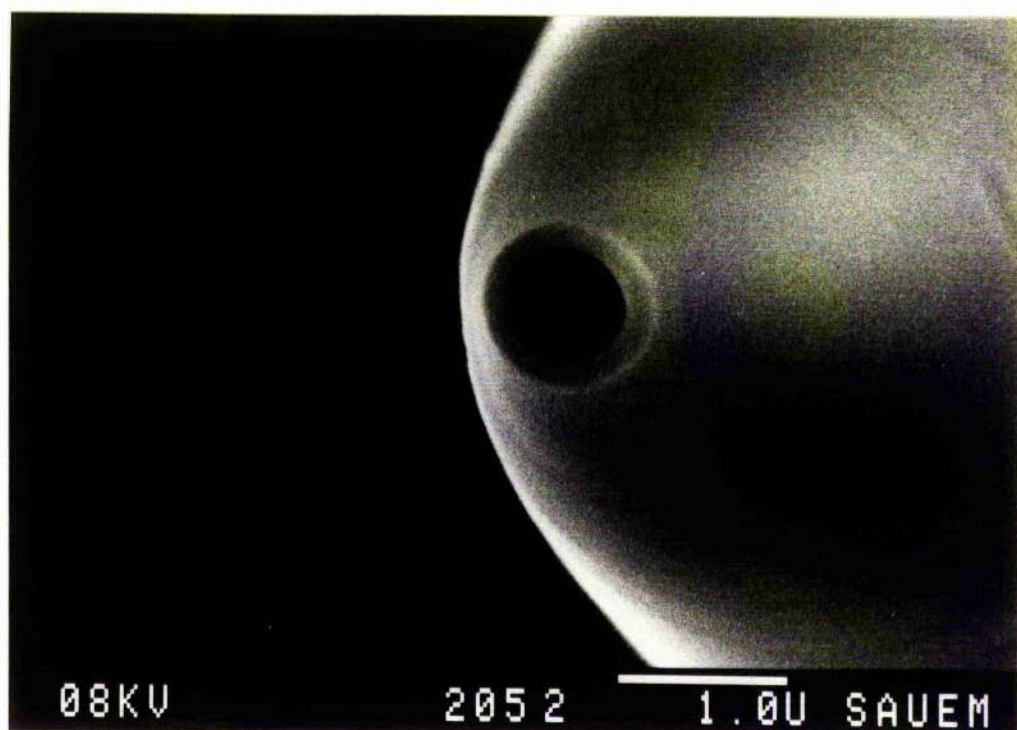
Figure 2.4b

A scanning electron micrograph of a heat polished patch pipette. Note the smooth appearance of the glass and the tip which enabled gigaohm seals to be obtained. From the micrographs there is a considerable difference between unpolished and polished patch pipettes. Polished patch pipettes have smaller tip diameters than unpolished patch pipettes.

a



b



up into the tip.

Backfilling of the pipettes was performed with thinned polythene tubing using filtered solutions. After backfilling, bubbles remained near the tip and these were carefully removed by tapping the pipette with the forefinger. Before pipettes were used they were quickly viewed at a magnification of 100 times to ensure cleanliness of the tip and solution.

## 2.13 PATCH CLAMP APPARATUS

Patch clamp recordings were performed using either an EPC-5 or an EPC-7 patch amplifier (List Electronic Darmstadt F.R.G.). A Ceptu stimulator was used to provide a 1V pulse to the patch amplifier, this pulse was used to measure pipette resistance and to monitor gigaseal formation. The neurones were visualized with an Olympus phase contrast microscope at a magnification of 200 times.

The pipette holder was attached to the amplifier headstage which was controlled coarsely in a vertical plane by a Palmer massive stand and finely by a Narishige MO-103N hydraulic micromanipulator. Pipettes for applying agents by pressure ejection were manoeuvred into position with a Prior micromanipulator. Experiments were monitored on line with a Gould type 220 oscilloscope and stored on a Racal 4DS FM tape recorder. The bath was grounded with a Ag/AgCl wire which was made by coating a Ag wire with molten silver chloride.



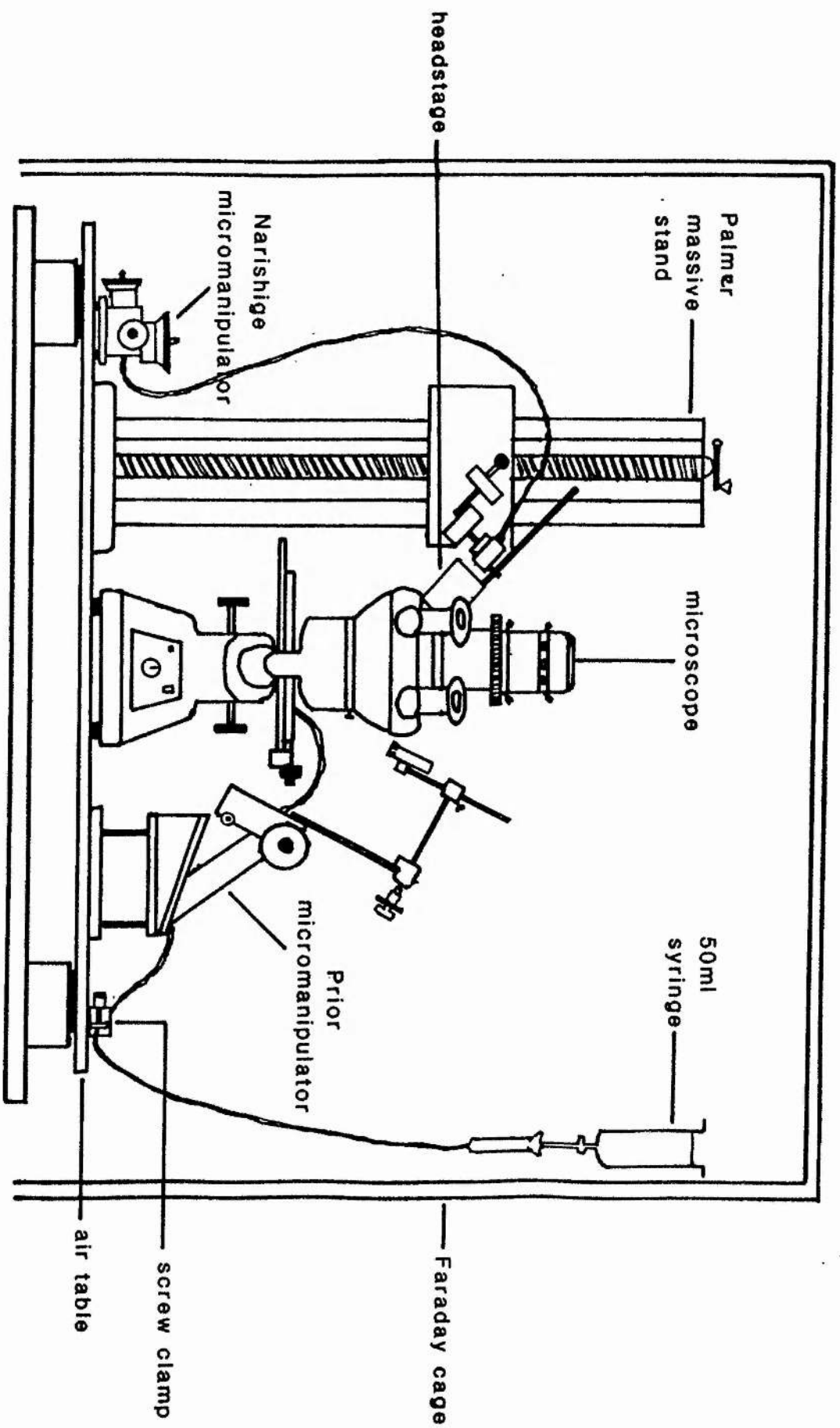
## 2.14 ARRANGEMENT OF APPARATUS FOR PATCH-CLAMP RECORDING

The microscope, manipulators and aspirator flasks were positioned on a steel baseplate, and enclosed in a copper Faraday cage. The microscope and the manipulators could act as aerials for electrical noise hence they were connected to a common ground along with the baseplate and Faraday cage. The high quality signal ground of the patch amplifier was employed as the common ground. For whole-cell experiments the front of the cage was not lowered however for single-channel experiments it was necessary to lower the front of the cage to reduce the background noise level to adequately resolve unitary currents.

A rectangular hole cut at the top of the cage above the reservoir enabled solutions to be introduced to the culture plate, while recordings were being made. The baseplate rested on a vibration isolation table (Ealing Laboratories). Pressure obtained from a high pressure gas cylinder was used to float the baseplate on four pockets of air at each corner. Pressures typically used to raise the baseplate and equipment were between 2-3 bars. The electronic equipment was kept on a rack to the side of the cage. Figure 2.5 shows a schematic representation of the arrangement of equipment upon the air table.

Figure 2.5

A schematic representation of the arrangement of equipment upon the baseplate. The 50ml syringe (reservoir) was used to perfuse the culture plate, the rate of perfusion could be adjusted using the plastic screw clamp. The aspirator flasks which were located behind the Palmer stand were omitted from the diagram for clarity. The equipment was surrounded by a copper Faraday cage.



## 2.15 CELL-ATTACHED PATCH FORMATION

Filled patch pipettes were firmly secured onto the pipette holder containing the Ag/AgCl wire which in turn was connected to the amplifier headstage. Positive or negative pressure could be applied to the pipette by mouth(or syringe) via a 1m length of 1mm diameter PVC tubing. As the pipette was lowered into the bath, positive pressure was applied to the pipette to prevent the accumulation of any debris at the tip. Once in the bath, the potential difference between the pipette and the bath was offset to zero. This adjusted potential served as reference for any changes in the patch potential. The approximate resistance of the pipette was measured by passing a 1V pulse from the stimulator to the amplifier and measuring the resulting current pulse. The amplitude of the current pulse was inversely proportional to the pipette resistance. Pipette resistances used varied from 1-7M $\Omega$  with high resistance pipettes being favoured for spinal neurones and low resistance pipettes being used for DRG neurones.

The patch pipette was lowered onto the selected cell, upon contact with the membrane, a sizeable reduction in the current pulse was observed, indicating the increased resistance proffered by the membrane. Slight suction (negative pressure) applied to the pipette resulted in further reduction of the pulse, until eventually the formation of a seal resistance greater than  $10^9$  G $\Omega$  (gigaohm) between the pipette and the membrane was formed. This configuration is the cell-attached patch mode of the patch clamp technique. From the cell-attached patch all the other configurations can be achieved.

## 2.16 WHOLE-CELL RECORDING

After formation of the cell-attached patch, the pipette potential was adjusted to -60mV which is the approximate resting potential of these neurones. The patch of membrane spanning the tip was ruptured by additional suction thus allowing electrical access to the inside of the cell. Whole-cell recording was indicated by an increase in the background current noise and invariably a slight shift in the current level.

In whole-cell recording, the cell is effectively dialyzed with the contents of the patch pipette (Fenwick, Marty and Neher, 1982). Often in the case of spinal neurones synaptic currents could be viewed at certain holding potentials. Using the current clamp mode of the patch amplifier, the resting membrane potential of the cell could be determined. Also, in current clamp and command modes depolarizing and hyperpolarizing current could be injected into the cell.

## 2.17 OUTSIDE-OUT PATCH FORMATION

Once having gained access to the inside of the cell with whole-cell recording, the patch pipette was slowly (over a period of seconds) withdrawn from the cell. The background noise consequently decreased and the resistance simultaneously increased to a value greater than a gigaohm. This sealing accompanying withdrawal of the pipette resulted from the formation of a new membrane bilayer at the pipette tip with its outside face facing the bath solution. This is the outside-out patch formation.

## 2.18 DATA COLLECTION AND ANALYSIS

The whole-cell clamp experiments were recorded on line with a Gould 220 chart recorder and also occasionally on magnetic tape using a Racal 4DS FM tape recorder. For whole-cell experiments, data was stored on magnetic tape played at  $\frac{15}{16}$  or  $1\frac{7}{8}$  inches/second. In the single-channel experiments data were recorded on tape played at either  $3\frac{3}{4}$  or  $7\frac{1}{2}$  inches/second. The filtering from the patch amplifier was 3kHz. With the tape played at these faster speeds the final effective filtering was 2.5kHz. Off line analysis of whole-cell and single-channel currents were performed using the movable cursors of the Nicolet 3091 digital oscilloscope. Single-channel currents could be expanded in both vertical and horizontal directions and using the movable cursors their amplitudes could be accurately measured.

An IBM PC AT computer was used to perform kinetic analysis of open and closed times, with software (PAT3) kindly donated by John Dempster. Prior to analysis, single-channel data were band limited with a low pass filter (1kHz) and digitized at 100 $\mu$ s per point with an analogue to digital (A/D) converter so that opening and closing transitions could be detected. A 50% threshold level between the closed and main open state was used to detect opening transitions. An opening event was deemed to have occurred once this threshold had been obtained. Transitions to other sub- and supra-conductance states were edited out of the final record along with multiple openings.

## 2.19 PRESSURE APPLICATION OF DRUGS

Drugs were ejected from pressure pipettes using regulated pressure applied to the open end of the pipette via a polyethylene tubing. The advantages of pressure ejection include:

(a) Known concentrations of drugs can be applied to neurones, thereby allowing a quantitative analysis of drug action.

(b) One can also determine whether the drug actions are occurring at physiological or therapeutic concentrations.

(c) The relatively rapid time course of application is important for drugs that are rapidly desensitizing.

(d) Drugs which act at very low concentrations and those that are relatively insoluble can be studied using pressure ejection.

(e) Drugs can be applied at a physiological pH, whereas with iontophoresis the drugs have acid or base added to provide the agent with a net positive or negative charge and this often results in large changes in pH.

Drugs were applied to cells and isolated outside-out patches using a Picospritzer (General Valve Corporation, USA) and a modified garden spray kit which was used to supply the pressure. Filamented borosilicate glass (Clark Electromedical GC150TF-15) was used to



produce pressure pipettes and these were pulled in a two stage process on the Narishige PP83 puller. The pressure pipettes were pulled in a manner identical to that used for patch pipettes. They had resistances of 1-1.5M $\Omega$  when filled with a physiological 140mM NaCl solution. Pressure pipettes were filled with filtered (0.22 $\mu$ m) solutions as described previously for patch pipettes.

The amount (volume) of solution released is almost invariably a linear function of applied pressure, or of the time for which constant pressure was applied (Sakai, Swartz and Woody, 1979). In these experiments the pressure was normally held at a constant value between 10-15 p.s.i. (70-105kPa). Hence the volume of solution released was directly proportional to the duration of the pulse. In most of the experiments durations, employed ranged from 20-100ms unless otherwise stated. With the whole-cell voltage clamp experiments, the picospritzer was triggered externally by either a Tektronix (type 161 pulse generator and 162 waveform generator) stimulator or a homemade stimulator. The duration and the pulse frequency on the stimulators could be varied at will.

In all whole-cell voltage clamp experiments the picospritzer was triggered at a frequency of 0.033Hz (once every 30 seconds). This frequency was used to minimize desensitization and helped monitor drug action on the whole-cell currents. The pressure pipette was positioned some 30-80 $\mu$ m away from the cell or isolated patch under study. Before each experiment the pressure pipette was tested by applying short test pulses to loose debris or cellular matter. If visible movement of the debris/cellular matter occurred, the pipette was deemed satisfactory. However, on occasions the tip of the pipette

became blocked . In this case, the pipette was discarded and replaced.

## 2.20 PERFUSION SYSTEM

A simple perfusion system was employed to expose the neurones to a continuous flow of physiological solution containing 140mM NaCl and drug containing solutions. The inlet and outlet tubes were made from bent filamented borosilicate glass as described for pressure pipettes. These tubes were connected by plastic tubing to the reservoir and outflow. The reservoir consisted of a 50ml syringe positioned approximately 30cm above the stage of the microscope. The perfusion rate could be regulated by means of a simple screw clamp located between the inlet and the reservoir. Perfusion rates were in the range 1-6ml/minute. Solutions were removed from the culture plate by suction using an electrical pump connected via rubber tubing to two aspirator flasks.

## 2.21 SOLUTIONS

In most of the whole-cell voltage clamp experiments the bath solution contained a normal physiological solution of 140mM NaCl; whereas the patch pipette contained either 140mM CsCl or 140mM KCl solutions. Using the above solutions, containing approximately equal  $\text{Cl}^-$  concentrations, the  $\text{Cl}^-$  equilibrium potential was  $\approx 0\text{mV}$ . In single-channel experiments, various different patch pipette solutions were employed including (a) 140mM CholineCl (b) 140mM TrisCl, (c) 25mM

CsCl, (d) 140mM KCl, (e) 140mM CsCl, (f) 300mM KCl.

All of the bath solutions used were initially filtered through a 1.6 $\mu$ m Whatman glass microfibre filter and then through a 0.22 $\mu$ m Millipore filter. This proved necessary to enable gigaseals to be obtained. Detailed compositions of the solutions are shown in Table 2.1.

The chloride activity ( $a_{Cl}$ ) of the main solutions used, namely 140mM NaCl, 140mM CsCl, and 25mM CsCl were estimated by interpolation from standard tables (Robinson and Stokes, 1959). This was done by calculating the ionic strength of a given solution and then this value was used to obtain the mean activity coefficient for the major salt. The mean activity coefficient was converted if necessary to give the activity for monovalent ions and this value used to obtain the  $a_{Cl}$  of the solution. These gave  $a_{Cl}$  of approximately 103mM and 105mM for the 140mM CsCl and 140mM NaCl solutions respectively. The low  $Cl^-$  (25mM CsCl) solution had an estimated  $a_{Cl}$  of 23mM. Essentially similar values were obtained from measurements of the above solutions with a Buchler digital Chloridometer. Permeability coefficients were calculated from the Goldmann Hodgmann and Katz (GHK) equation and used chloride activities  $a_{Cl}$  of the solutions concerned.

Table 2.1

Composition of the solutions used in both the bath and patch pipette. All concentrations shown are in mM, the pH was adjusted to 7.2 with the addition of KOH, CsOH or NaOH. The total  $\text{Cl}^-$  concentration is shown at the bottom of each solution in mM.

### BATH SOLUTIONS

#### PHYSIOLOGICAL (140 NaCl) SOLUTION

140 NaCl  
1 MgCl<sub>2</sub>  
1 CaCl<sub>2</sub>  
1 KCl<sub>2</sub>  
10 HEPES  
[Cl] = 145

#### EXTERNAL TRIS SOLUTION

140 TrisCl  
1 MgCl<sub>2</sub>  
1 CaCl<sub>2</sub>  
1 KCl<sub>2</sub>  
10 HEPES  
[Cl] = 145

### PATCH PIPETTE SOLUTIONS

#### 25 CsCl SOLUTION

25 CsCl  
55 CS<sub>2</sub>SO<sub>4</sub>  
55 sucrose  
1 CaCl<sub>2</sub>  
1 MgCl<sub>2</sub>  
1 KCl<sub>2</sub>  
11 EGTA  
10 HEPES  
[Cl] = 30

#### 140 CHOLINECl SOLUTION

140 CholineCl  
1 MgCl<sub>2</sub>  
11 EGTA<sub>2</sub>  
10 HEPES  
[Cl] = 142

#### INTERNAL 140 TRIS SOLUTION

140 TrisCl  
3 KCl  
1 MgCl<sub>2</sub>  
11 EGTA<sub>2</sub>  
10 HEPES  
[Cl] = 145

#### 140 CsCl SOLUTION

140 CsCl  
1 MgCl<sub>2</sub>  
11 EGTA<sub>2</sub>  
10 HEPES  
[Cl] = 142

#### 140 KCl SOLUTION

140 KCl  
3 NaCl  
1 MgCl<sub>2</sub>  
11 EGTA<sub>2</sub>  
10 HEPES  
[CL] = 145

#### 300 KCl SOLUTION

300 KCl  
15 NaCl  
2 MgCl<sub>2</sub>  
2 EGTA<sub>2</sub>  
100 HEPES  
[Cl] = 319

## 2.22 LIQUID JUNCTION POTENTIALS

Where differences in the ionic composition exist between the pipette and the bath solution, a correction has to be applied for the junction potential. This liquid junction potential is present when the pipette current is offset before sealing onto the cell, but disappears once the seal is made. Junction potentials were measured by comparing the zero current voltage in symmetrical solutions and after replacing the bath solution with the solution to be tested (Fernandez, Fox and Krasne, 1984). During this procedure, a 1M KCl agar bridge was used in the bath to eliminate changes in the reference electrode potential.

With the pipette containing a 140mM KCl solution and the bath a 140mM NaCl solution, a liquid junction potential of 2mV was observed, which is similar to that observed by Fenwick Marty and Neher in 1982. With a low  $\text{Cl}^-$  solution (25mM CsCl) in the pipette and 140mM NaCl in the bath a 4mV liquid junction potential was found, whereas with 140mM TrisCl in the bath the potential was 6mV. The corrected potential value was the measured potential minus the liquid junction potential in outside-out and whole-cell recording mode. Since the liquid junction potentials were small with 140mM KCl/140mM NaCl solutions they were neglected. However liquid junction potentials were corrected for with 25mM CsCl in the patch pipette, in both outside-out and whole-cell recording mode.

## 2.23 DRUGS

All drugs used in this thesis were applied to cells and isolated outside-out patches in a physiological bath solution of 140mM NaCl. The endogenous and synthetic steroids, as well as diazepam were initially dissolved in 1ml of 95% ethanol at concentrations between 10-50mM, and then diluted to the required concentration into the bath solution. Phenobarbitone sodium was dissolved in 1ml of distilled water and diluted to 500µM with physiological solution containing 140mM NaCl. GABA, bemegride, and pentobarbitone were dissolved directly into the bath solution. Bicuculline was dissolved in 10mM HCl at a concentration of 100mM and diluted down to the required concentration.

The steroids 5 $\beta$ pregnane-3 $\alpha$ ol-20-one (pregnanolone) and 5 $\beta$ pregnane-3 $\alpha$ -20-dione (pregnanedione) were obtained from Sigma along with GABA, pentobarbitone sodium and + bicuculline. Alphaxalone and betaxalone were gifts from Glaxo. Diazepam was a gift from Roche UK. Phenobarbitone sodium and bemegride were obtained from BDH.



## RESULTS

## RESULTS

### 3.1 VOLTAGE RECORDINGS OF MOUSE SPINAL AND RAT DRG NEURONES

Experiments were conducted on isolated mouse spinal neurones and rat DRG neurones maintained in culture. Mouse spinal and rat DRG neurones were identified on morphological grounds, as described in the methods. Further evidence to substantiate the cell type was made by using the current clamp mode of the patch amplifier after having achieved whole-cell conditions (see below 3.1.1 and 3.1.2).

#### 3.1.1 MOUSE SPINAL NEURONES

Spinal cord neurones in culture developed a dense network of processes with functioning synapses. Spinal neurones showed spontaneous inhibitory and excitatory synaptic potentials. The latter often triggering action potentials. A variety of spontaneous electrical activity was observed in spinal neurones ranging from simple repetitive action potential firing to complex firing, that included periods of quiescence and sustained depolarizations.

Figure 3.1 (a and b) shows the spontaneous electrical activity of two different spinal neurones. It should be noted that the action of the inhibitory neurotransmitters GABA and glycine on these cells at the resting membrane potential would be depolarizing, because of the high  $\text{Cl}^-$  concentration within the patch pipette (Mathers, 1985). Thus



inhibitory postsynaptic potentials would appear as depolarising potentials.

Intracellular injection of depolarizing current into spinal neurones has been shown to result in increased action potential firing (Bigalke, Dreyer and Bergey, 1985). Several workers have used mouse spinal neurones to test the effects of various drugs on the membrane currents which underly impulse activity. These drugs include phenytoin (McLean and MacDonald, 1983); botulinum A neurotoxin (Bigalke et al., 1985); ethosuximide (McLean and MacDonald, 1986a); carbamazepine and 10,11 epoxycarbamazepine (McLean and MacDonald 1986b); and flurazepam (MacDonald and Barker, 1982). The action potentials of spinal neurones have both sodium and calcium components (Heyer and MacDonald, 1982). In the present work, the resting membrane potential of spinal neurones varied considerably from cell to cell, the mean was  $-54.80 \pm 7.65$  (mean  $\pm$  S.D.)  $n=21$ .

### 3.1.2 RAT DRG NEURONES

Rat DRG neurones at resting membrane potential do not normally fire action potentials (Ford and Kelly, 1985). DRG neurones in other species have similar electrical properties, for example, mouse DRG neurones are also electrically silent at resting potential. However, they can be made to fire upon the injection of depolarizing current (Ransom et al., 1977; Simmoneau, Distasi, Tauc and Barbin, 1987; Heyer and MacDonald, 1982).

A voltage recording from a rat DRG neurone which did not fire action potentials at resting level, is shown in figure 3.2, however, upon

injecting depolarizing current, the cell fired action potentials. The frequency of firing increased as more current was injected into the cell. The resting membrane potential of the DRG neurones recorded in this study was  $-57.68 \pm 7.19\text{mV}$  (mean  $\pm$  sd)  $n = 25$ .

### 3.2 BASIC PHARMACOLOGY OF GABA-EVOKED WHOLE-CELL CURRENTS

Application of a brief pulse of GABA (10-100 $\mu\text{M}$ ) to spinal neurones voltage clamped at negative holding potentials, resulted in the activation of a transient membrane current similar to that seen in the top trace of figure 3.3. Bicuculline, a phthalide isoquinoline alkaloid is the most widely used GABA<sub>A</sub> antagonist. Its antagonistic properties were discovered in 1970 as the result of a systematic study of convulsant alkaloids (Curtis, Duggan, Felix and Johnston, 1970). Using local administration with iontophoresis, bicuculline antagonized the neuronal action of GABA on most CNS neurones and some peripheral neurones (Johnston, 1978). Bicuculline is a competitive antagonist of the GABA<sub>A</sub> receptor within neurones of the rat cuneate nucleus (Simmonds, 1978, 1980).

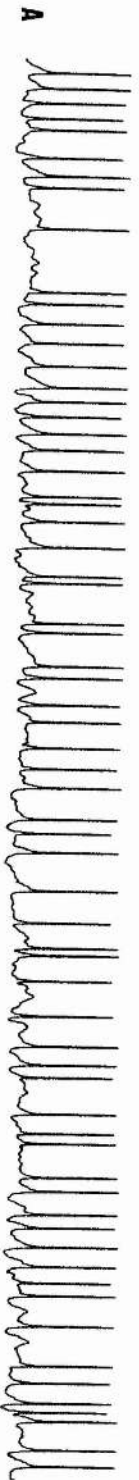
An example of the reversible suppression of GABA-evoked whole-cell currents by bicuculline in a spinal neurone is shown in figure 3.3. Bicuculline (2-10 $\mu\text{M}$ ) was capable of almost completely blocking the GABA-evoked currents in 6 neurones. GABA-evoked whole-cell currents in rat DRG neurones were also blocked by bicuculline. Figure 3.4 shows an example of the reversible suppression of GABA currents by 10 $\mu\text{M}$  bicuculline; in 6 DRG neurones, bicuculline virtually abolished the GABA response.

Figure 3.1

Voltage recordings from spinal neurones.

a: A spinal neurone maintained in culture for 35 days showing action potentials. The small depolarising potentials may be synaptic potentials. The resting membrane potential of this cell was  $-43\text{mV}$ .

b: A spinal neurone maintained in culture for 31 days showing complex synaptic activity with sustained depolarizations. These depolarizations led to action potential firing. The resting membrane potential was  $-56\text{mV}$ . The patch pipette contained an intracellular physiological solution of  $140\text{mM}$  KCl in both cases. The size of the action potentials were attenuated by the frequency characteristics of the chart recorder.



20mV  
500ms

Figure 3.2

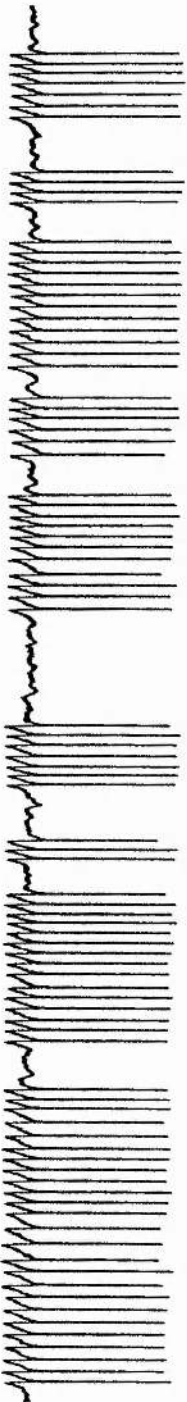
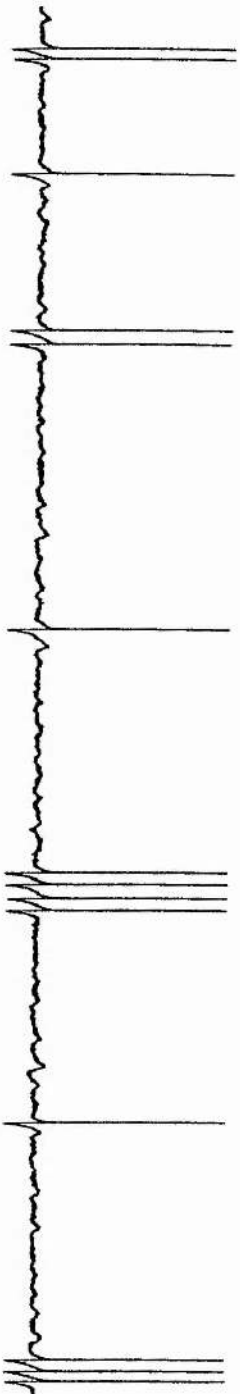
Voltage recordings from a rat DRG neurone maintained in culture for 3 days.

The top trace shows a recording from a neurone at resting potential and the lack of any spontaneous synaptic potentials. The resting potential was  $-64\text{mV}$ .

The middle trace shows the effect of injecting depolarizing current sufficient to depolarize the cell to approximately  $-50\text{mV}$ . Infrequent action potentials were observed.

The lower trace shows the cell depolarized to approximately  $-43\text{mV}$  by injecting further current. The frequency of action potential firing was increased compared to the middle trace.





25mV  
500ms

Figure 3.3

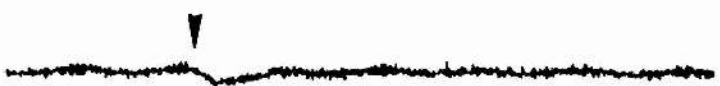
Antagonistic effect of 5 $\mu$ M bicuculline on whole-cell inward currents evoked by 20 $\mu$ M GABA in a mouse spinal neurone. The suppression of the current in the middle trace was reversed upon washing (bottom trace). The cell was voltage clamped at -60mV. The patch pipette contained a 140mM KCl solution and the bath contained a physiological solution of 140mM NaCl.

For details of application of drugs-see Methods

CONTROL



5 $\mu$ M BICUCULLINE



WASH



500pA  
2s

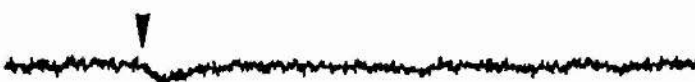
Figure 3.4

The antagonistic effect of  $10\mu\text{M}$  bicuculline on whole-cell inward currents evoked by  $30\mu\text{M}$  GABA in a rat DRG neurone. Application of GABA is shown by the arrows. The effect of bicuculline was reversed upon washout (bottom trace). The cell was voltage clamped at  $-60\text{mV}$ . The patch pipette contained a  $140\text{mM}$  CsCl solution, and the bath contained a physiological solution of  $140\text{mM}$  NaCl.

CONTROL



10 $\mu$ M BICUCULLINE



WASH



100pA  
1s

Barbiturates have been previously reported to enhance GABA action in mammalian neurones (Eccles and Malcolm, 1946; Schultz and MacDonald, 1981; Nicoll, Eccles, Oshima and Rubin, 1975). Barker and McBurney in 1979 showed that the anticonvulsant barbiturate phenobarbitone potentiated GABA-evoked current responses in mouse spinal neurones.

The amplitude of GABA-evoked whole-cell currents was increased by phenobarbitone. A similar potentiating action of 500 $\mu$ M phenobarbitone on GABA-evoked currents is shown in figure 3.5. In 5 neurones 500 $\mu$ M phenobarbitone in the bathing solution potentiated GABA-evoked currents by  $177 \pm 17.93\%$  (mean  $\pm$  S.E.). All quantitative results are presented below in the form mean  $\pm$  standard error of the mean (mean  $\pm$  S.E.) number of cells tested (n = x) unless otherwise stated.

Figure 3.5

The reversible potentiating effect of 500 $\mu$ M phenobarbitone on the inward current evoked by 20 $\mu$ M GABA in a mouse spinal neurone. Local application of GABA is shown by the arrows; the cell was voltage clamped at -70mV. The patch pipette contained a 140mM KCl solution and the bath contained a physiological solution of 140mM NaCl.



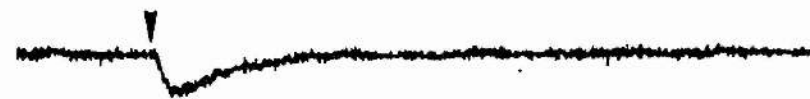
CONTROL



500  $\mu$ M  
PHENOBARBITONE



WASH



500pA  
1s

### 3.3 IONIC MECHANISM OF THE GABA RESPONSE (WHOLE-CELL STUDIES)

#### 3.3.1 MOUSE SPINAL NEURONES

GABA<sub>A</sub> receptors in the mammalian CNS are usually coupled to Cl<sup>-</sup> selective channels (Curtis, Hosli, Johnston and Johnston, 1968; Krnjevic, Kelly, Morris and Yim, 1969). The ionic mechanism of the GABA response in spinal neurones was investigated using different Cl<sup>-</sup> concentrations within the patch pipette. A 140mM CsCl solution was employed in the patch pipette to suppress unwanted K<sup>+</sup> conductances. The total Cl<sup>-</sup> concentration in the patch pipette was 142mM, while the bath contained a physiological solution containing 140mM NaCl, with a total Cl<sup>-</sup> concentration of 145mM. Using the Cl<sup>-</sup> activities of the above solutions, the calculated Cl<sup>-</sup> equilibrium potential was close to 0mV ( $E_{Cl} = -0.48mV$ )

Brief pressure application of GABA to the soma of spinal neurones held at a range of holding potentials ( $V_h$ ) evoked transient membrane currents. Figure 3.6a shows the effect of different holding potentials on the size and sign of the currents evoked by GABA. Outward currents were evoked by GABA at positive holding potentials, while inward currents were evoked at negative holding potentials. The relationship between the size and sign of the currents and the holding potential is shown in figure 3.6b. The GABA-evoked currents reversed in direction at a holding potential of approximately 0mV, the mean being  $-3.6 \pm 1.12mV$  ( $n=14$ ).

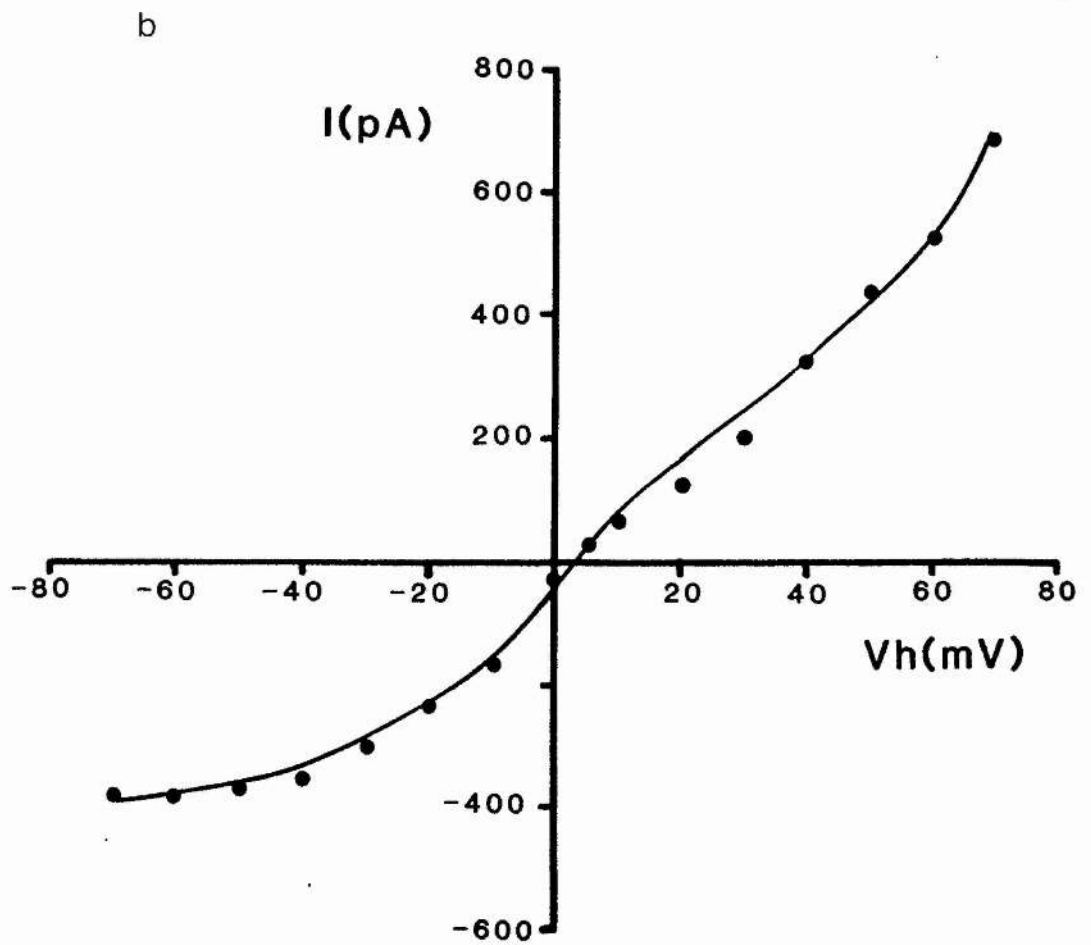
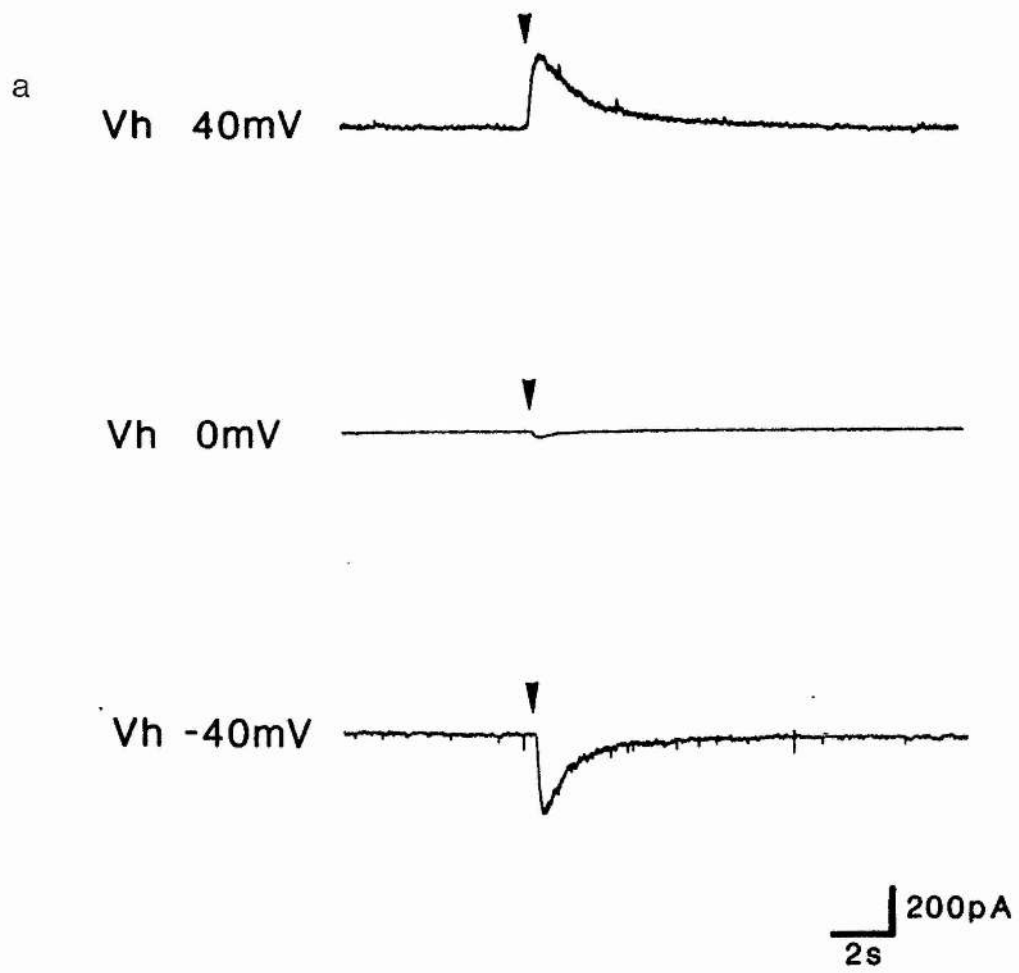
The steady state whole-cell I-V relationship shown in figure 3.6b showed outward rectification. Outward rectification caused by GABA has been observed by several workers (Bormann, Hamill and Sakmann, 1987; Akaike, Inoue and Krishtal, 1986; Cottrell, Lambert and Peters, 1985; Weiss, Barnes and Hablitz, 1988). This outward rectification presumably reflects a voltage dependence of the GABA gating reaction since the instantaneous I-V relation was linear (Bormann et al., 1987). To support the notion that GABA activated a  $\text{Cl}^-$  selective conductance, the high  $[\text{Cl}^-]$  in the patch pipette was replaced with a low  $[\text{Cl}^-]$  solution as used by Hamill, Bormann and Sakmann in 1983. This low  $\text{Cl}^-$  solution had a total  $\text{Cl}^-$  activity of 23mM. The bath solution remained a physiological solution containing 140mM NaCl ( $\alpha_{\text{Cl}}$  of 105mM). With these solutions the calculated  $E_{\text{Cl}}$  was -38mV.

Whole-cell voltage clamp recordings were performed using these asymmetrically distributed  $\text{Cl}^-$  solutions. Local application of GABA to spinal neurones resulted in transient membrane currents as shown in figure 3.7. At a high negative holding potential ( $V_h = -88\text{mV}$ ) an inward current was observed. At a holding potential of -15mV an outward current was evoked, which became larger in size at a holding potential of +45mV. The graph in the figure shows the relationship between the holding potential and the size and the sign of the currents evoked by GABA. The mean reversal potential for the GABA-evoked currents was  $-35 \pm 2.2\text{mV}$  ( $n=9$ ).

Figure 3.6

(a) Effect of different holding potentials( $V_h$ ) on the currents evoked by pressure application of 20 $\mu$ M GABA (shown by arrows) in a mouse spinal neurone.

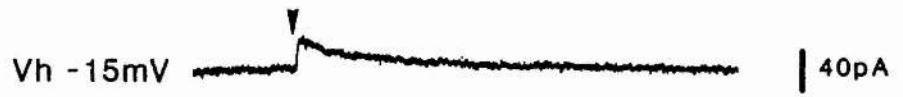
(b) The graph shows the relationship between the holding potential and the size and sign of the current evoked by local application of GABA. The patch pipette contained a 140mM CsCl solution to suppress unwanted potassium conductances, the bath contained a physiological solution of 140mM NaCl.



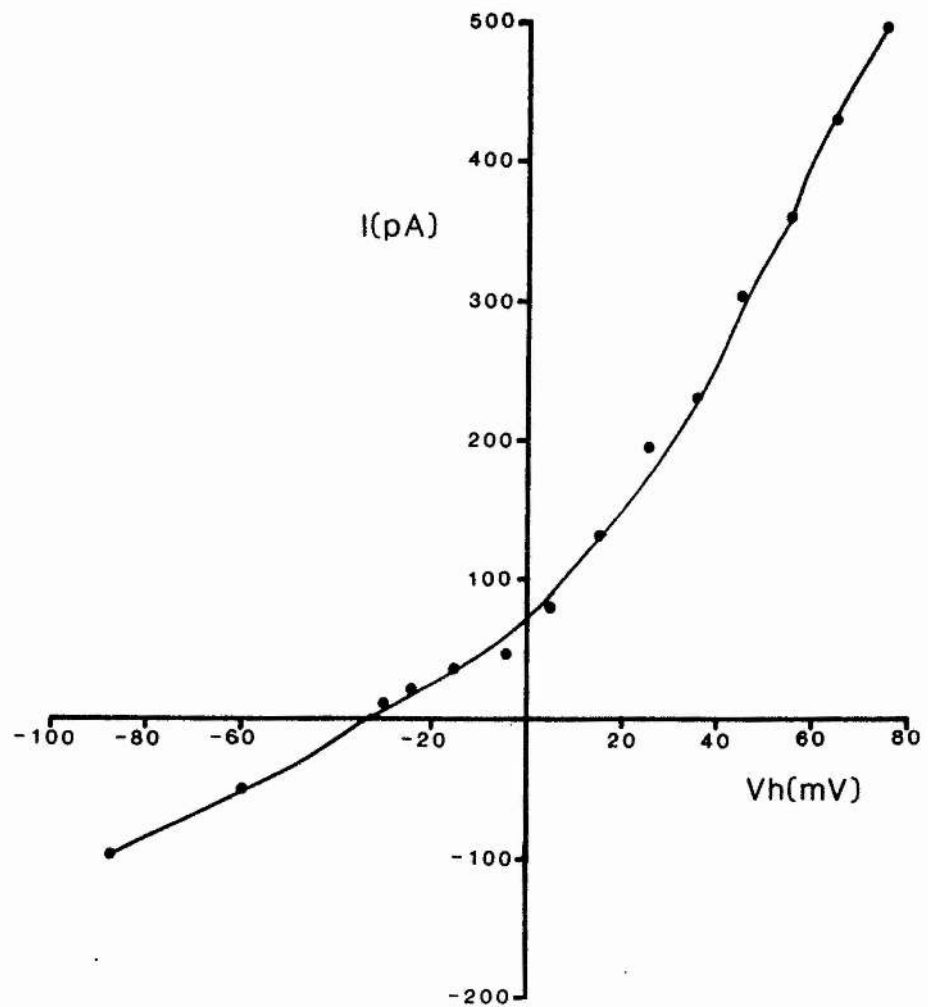
### Figure 3.7

The effect of different holding potentials on the currents evoked by pressure application of 10 $\mu$ M GABA (shown by arrows) in a mouse spinal neurone. The patch pipette contained a low Cl<sup>-</sup> solution of 25mM CsCl, while the bath contained a physiological solution of 140mM NaCl.

The graph below represents the relationship between the holding potential and the size and the sign of the currents evoked by GABA



2s





### 3.3.2 RAT DRG NEURONES

The protocol described above for spinal neurones was also applied to rat DRG neurones. Similar currents to those observed for spinal neurones were obtained. Figure 3.8 shows the transient currents evoked by GABA in symmetrical  $\text{Cl}^-$  solutions at different holding potentials. Inward currents were evoked at negative holding potentials, outward currents at positive potentials, with reversal of the response occurring at approximately 0mV. The mean reversal potential was  $-0.14 \pm 1.47\text{mV}$  ( $n=9$ ). Outward rectification was again observed.

Experiments were also performed using the low  $[\text{Cl}^-]$  in the patch pipette. Figure 3.9 shows GABA-evoked currents at different holding potentials in asymmetrical  $\text{Cl}^-$  solutions. The graph in the figure shows the relationship between the holding potential and the size and the sign of the currents evoked by GABA. The reversal of the response occurred at  $-38\text{mV}$ . With 9 cells the mean reversal potential was  $-34.77 \pm 1.95\text{mV}$ . These results described above suggest that application of GABA to rat DRG and mouse spinal neurones predominantly evoked a  $\text{Cl}^-$  selective conductance.

Control experiments in which physiological solution containing 140mM NaCl, from which GABA was omitted, was pressure applied to spinal neurones were performed. In 10 spinal neurones held at a range of holding potentials under whole-cell conditions, pressure application of physiological solution did not result in any transient membrane currents similar to that seen for GABA application. Durations and pressures employed for the ejection of physiological

Figure 3.8

The effect of different holding potentials on the currents evoked by 30 $\mu$ M GABA in a rat DRG neurone. Pressure application of GABA is indicated by the arrows.

The graph shows the relationship between the holding potential and the size and sign of the currents evoked by GABA. The patch pipette contained a 140mM CsCl solution, while the bath contained a physiological solution of 140mM NaCl.

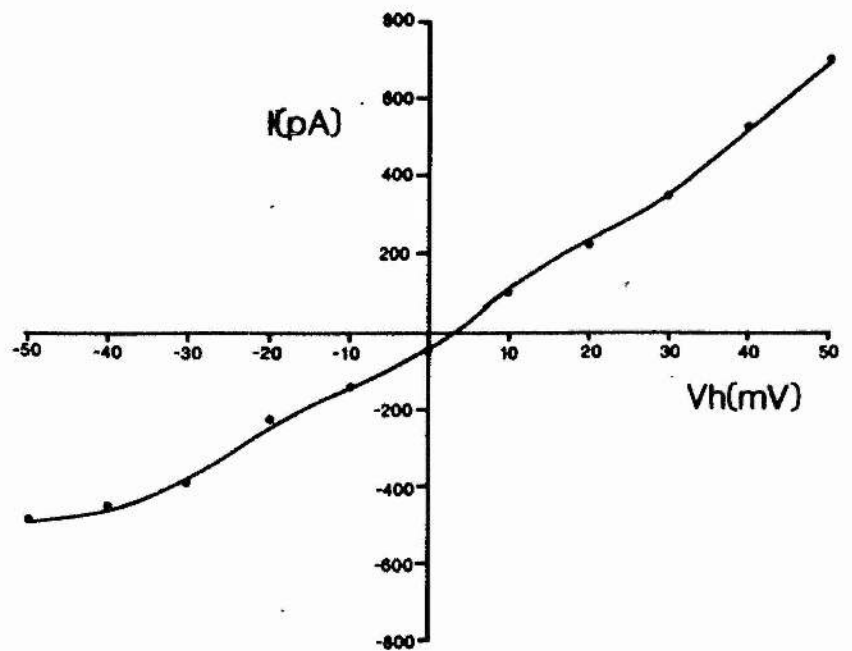
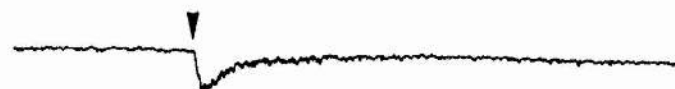


Figure 3.9

The effect of 3 different holding potentials on the currents evoked by 30 $\mu$ M GABA in a rat DRG neurone. The arrows indicate where GABA was applied; the patch pipette contained a 25mM CsCl solution and the bath contained a physiological solution of 140mM NaCl.

The relationship between the holding potential and the size and the sign of the currents evoked by GABA is shown in the graph

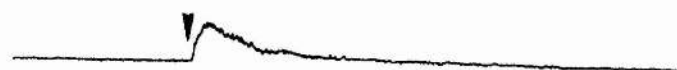
Vh -86mV



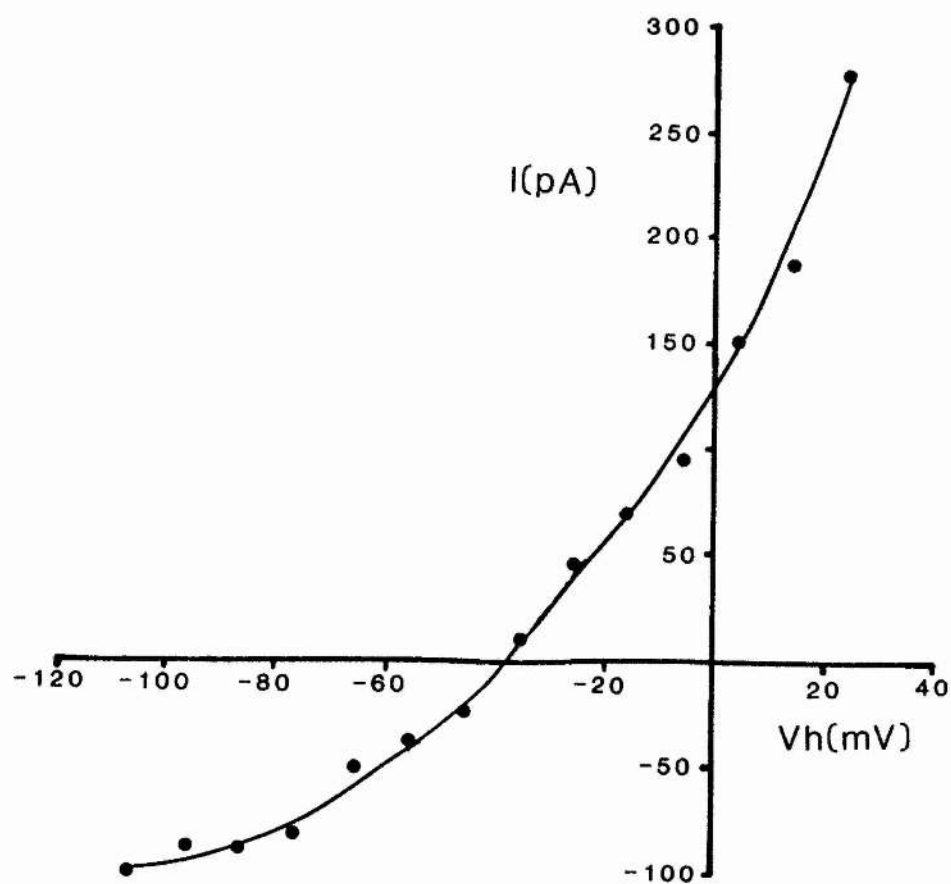
Vh -46mV



Vh -06mV



100pA  
2s



solution were similar to those used for GABA application (20-100ms). Longer durations were also used on some cells. However, still no evidence for the activation of any membrane currents were obtained. The lack of effect of applying physiological solution to a spinal neurone at different holding potentials is shown in figure 3.10. These results indicate that the currents evoked by pressure application of physiological solution containing GABA are indeed GABA-activated currents and are not due to pressure artifacts.

### 3.4 SPONTANEOUS UNITARY CURRENTS FROM MOUSE SPINAL NEURONES

#### 3.4.1 Spontaneous unitary currents in symmetrical $\text{Cl}^-$ solutions.

To study the actions of GABA at the single-channel level, the outside-out patch configuration of the patch clamp technique was used. The outside-out patch is particularly suited to the study of transmitter activated channels since known concentrations of transmitter and other drugs can be readily applied to these patches. Virtually all outside-out patches from the somato of mouse spinal neurones displayed inward unitary currents upon isolation at patch potentials of around -60mV. Symmetrically distributed  $\text{Cl}^-$  solutions were employed with the patch pipette on most occasions containing an intracellular solution of mainly 140mM CsCl. The Cs was used to suppress  $\text{K}^+$  currents and hence obtain I-V relationships for the unitary currents. The bath contained a physiological solution of 140mM NaCl. These spontaneous unitary currents had various different

Figure 3.10

The lack of effect of pressure application of physiological solution containing 140mM NaCl on a spinal neurone held in whole-cell voltage clamp conditions.

The top trace shows the cell held at -60mV, the arrows show 3 applications of physiological solution to the cell of duration 100, 400 and 800ms respectively.

The middle trace shows applications of 800 and 100ms at a holding potential of 0mV.

The bottom trace shows the same cell held at +50mV. The arrows indicate the application of physiological solution at durations of 100, 400 and 800ms respectively.

The patch pipette contained a 140mM CsCl solution, while the bath contained a physiological solution of 140mM NaCl.

$V_h - 60\text{mV}$



$V_h \ 0\text{mV}$



$\begin{array}{l} \text{└─ 400pA} \\ \text{2s} \end{array}$

$V_h + 50\text{mV}$





Generally these currents had brief opening events but they also displayed longer lasting openings from time to time. The I-V relationships for the spontaneous currents were obtained from the longer lasting and more active outside-out patches.

An example of the spontaneous current activity in an outside-out patch and its I-V relationship is shown in figure 3.11. The unitary current amplitudes were linearly related to the patch potential ( $V_p$ ) and had an interpolated reversal potential of 0mV. The slope conductances obtained from the I-V relationship were 21, 30 and 44pS for this particular outside-out patch. The conductances of these channels are very similar to those described by Hamill Bormann and Sakmann in 1983. These workers obtained conductance values of 19, 30 and 45pS for the glycine receptor and 20 and 30pS for the GABA receptor in mouse spinal neurones (please see Discussion).

Permeability values for the three conductance states shown in the figure were obtained using the data points, the Goldman Hodgkin Katz (GHK) equation and intracellular and extracellular  $Cl^-$  activities of 103 and 105mM respectively (see Appendix). The mean permeability values were  $5.65 \times 10^{-14} \text{ cm}^3 \text{ s}^{-1}$ ,  $7.75 \times 10^{-14} \text{ cm}^3 \text{ s}^{-1}$ , and  $10.20 \times 10^{-14} \text{ cm}^3 \text{ s}^{-1}$  for the 20pS, 30pS and 44pS states respectively. The number of unitary current amplitudes, along with the dominant conductance state varied from patch to patch. The frequency of the opening events also varied widely from patch to patch, but within patches there was neither a progressive decline of the activity nor periods of enhanced activity. Some of the excised outside-out patches were deliberately moved well away and across from the cells from which they were obtained. A few isolated patches were also exposed to

Figure 3.11

Spontaneous unitary currents observed in an outside-out patch isolated from the soma of a mouse spinal neurone. Different sizes of unitary currents were observed in this patch.

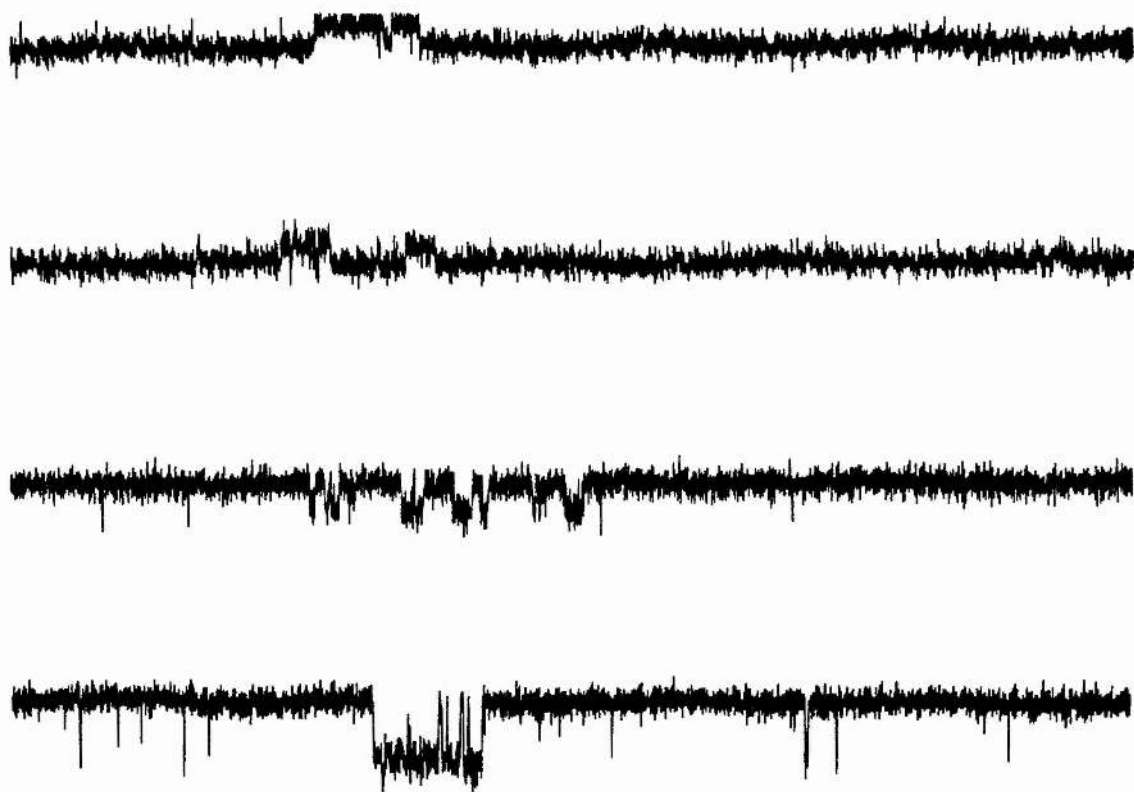
The top trace shows unitary currents of slope conductance 45pS at a patch potential of +40mV.

The second trace shows unitary currents of slope conductance 30pS at the same patch potential of +40mV.

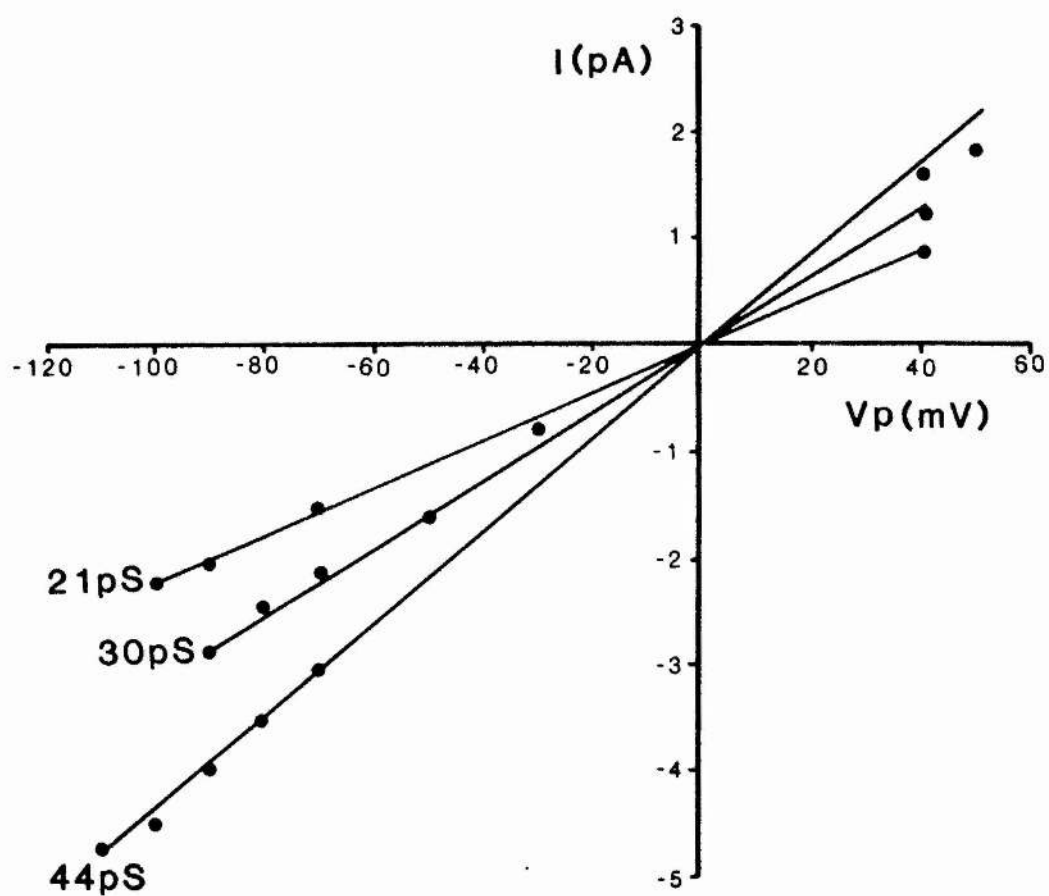
The third trace shows unitary currents of conductance 21pS at a patch potential of -70mV.

The bottom trace shows an example of unitary currents of slope conductance 45pS at a patch potential of -90mV.

The graph below shows the I-V relationship for the unitary currents described above. The amplitudes of the unitary currents were linearly related to the patch potential. This particular outside-out patch showed three distinct single channel current levels having slope conductances of approximately 21, 31 and 44pS and an interpolated reversal potential of 0mV. The patch pipette contained a 140mM CsCl solution and the bath contained a physiological solution of 140mM NaCl.



2pA  
50ms



physiological solution containing 140mM NaCl at rates of 1-3ml per minute. However, despite these manipulations no conspicuous difference in the spontaneous current activity of the patches was found.

Sometimes large spontaneous unitary currents having conductances greater than 45pS as determined from their I-V relationship were observed in isolated outside-out patches. These large conductance states generally tended to have brief opening events and were usually very infrequent. An example of such large conductance events and their I-V relationship is shown in figure 3.12 and figure 3.13 respectively. The outside-out patch described in figure 3.12 displayed a large conductance event of approximately 100pS. This state was open for long periods, and often showed transitions to various smaller conductance levels (shown by arrows). Apart from having this large conductance state other smaller conductance levels were also seen of 29, 43, and 50pS. Occasionally a very small conductance level event of 6-8pS was also found on this patch. From the graph, the I-V relationship was linear and again had an interpolated reversal potential of approximately 0mV.

#### 3.4.2 Lack of effect of cations on spontaneous unitary currents.

To help characterize the ionic selectivity of these spontaneous unitary currents, different patch pipette solutions were employed. These intracellular solutions contained mainly 140mM KCl or 140mM TrisCl. An extracellular solution containing mainly 140mM TrisCl was also on occasions used in the bath. No significant differences in the

Figure 3.12

Spontaneous unitary currents observed in an isolated outside-out patch from a mouse spinal neurone. The patch pipette contained a solution of 140mM CsCl, while the bath contained a physiological solution of 140mM NaCl.

The numbers on the left of each trace represents the patch potential in mV.

A large inwardly directed channel was seen at negative patch potentials; this channel had a slope conductance of 100pS. This large channel was open for long periods and often showed transitions to various different substates (shown by arrows).

Trace 1 shows transitions to subconductance levels of 63 and 87pS.

Trace 2 shows transitions to subconductance levels of 43 and 65pS

Trace 3 shows examples of subconductance levels to 71 and 43pS.

Apart from this large conductance channel other smaller single channel currents were also observed.

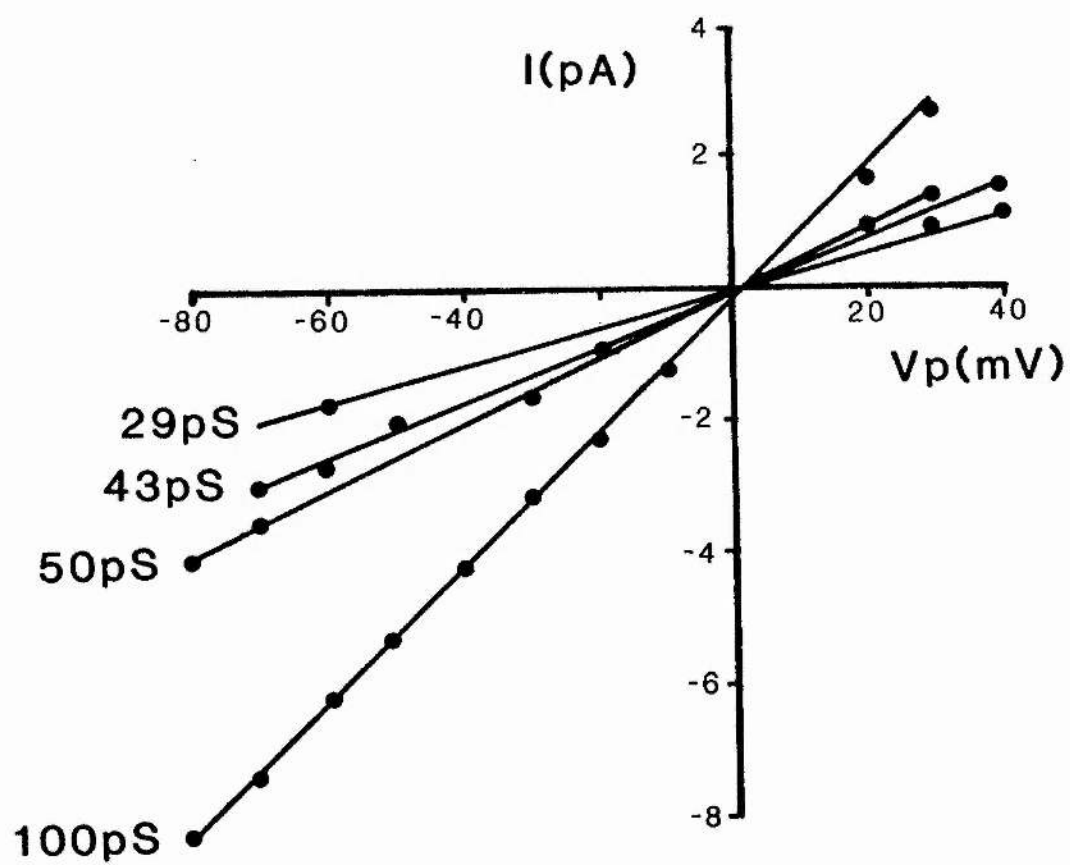
Trace 4 shows a channel of conductance 29pS

Trace 5 shows a channel of conductance 43pS, while the arrow shows a smaller conductance channel of 8pS. The last trace shows channel conductances of 50pS (indicated by the arrows).



Figure 3.13

The I-V relationship for the spontaneous currents described in figure 3.12. Unitary current amplitudes were linearly related to the patch potential ( $V_p$ ). Four different conductance states of 29, 43, 50 and 100pS were present. The interpolated reversal potential was 0mV.





type of spontaneous current activity was observed using these solutions. Multiple conductance states were again present in these patches. The large impermeant cation Tris was used since the spontaneous events could possibly be attributed to a non-selective cation channel which was equally permeable to  $\text{Cs}^+$  and  $\text{Na}^+$ .

Outside-out patches were obtained with solutions containing mainly 140mM TrisCl on one or both sides of the membrane patch. Figure 3.14 shows examples of spontaneous single channel currents and their I-V relationship in an outside-out patch with the patch pipette containing a solution of mainly 140mM TrisCl. This particular patch showed 5 distinct unitary current levels ranging in conductance from 19-42pS. The unitary current amplitudes were linearly related to patch potential and had an interpolated reversal potential of 0mV perhaps indicating  $\text{Cl}^-$  selective events. Some of the conductance states observed were more frequent than others and this is indicated by the number of data points in the I-V relationship. A total of 13 outside-out patches were obtained with Tris as the main cation on one or both sides of the membrane patch and all of these displayed spontaneous current activity which reversed at 0mV. I-V relationships were constructed from the longer lasting patches.

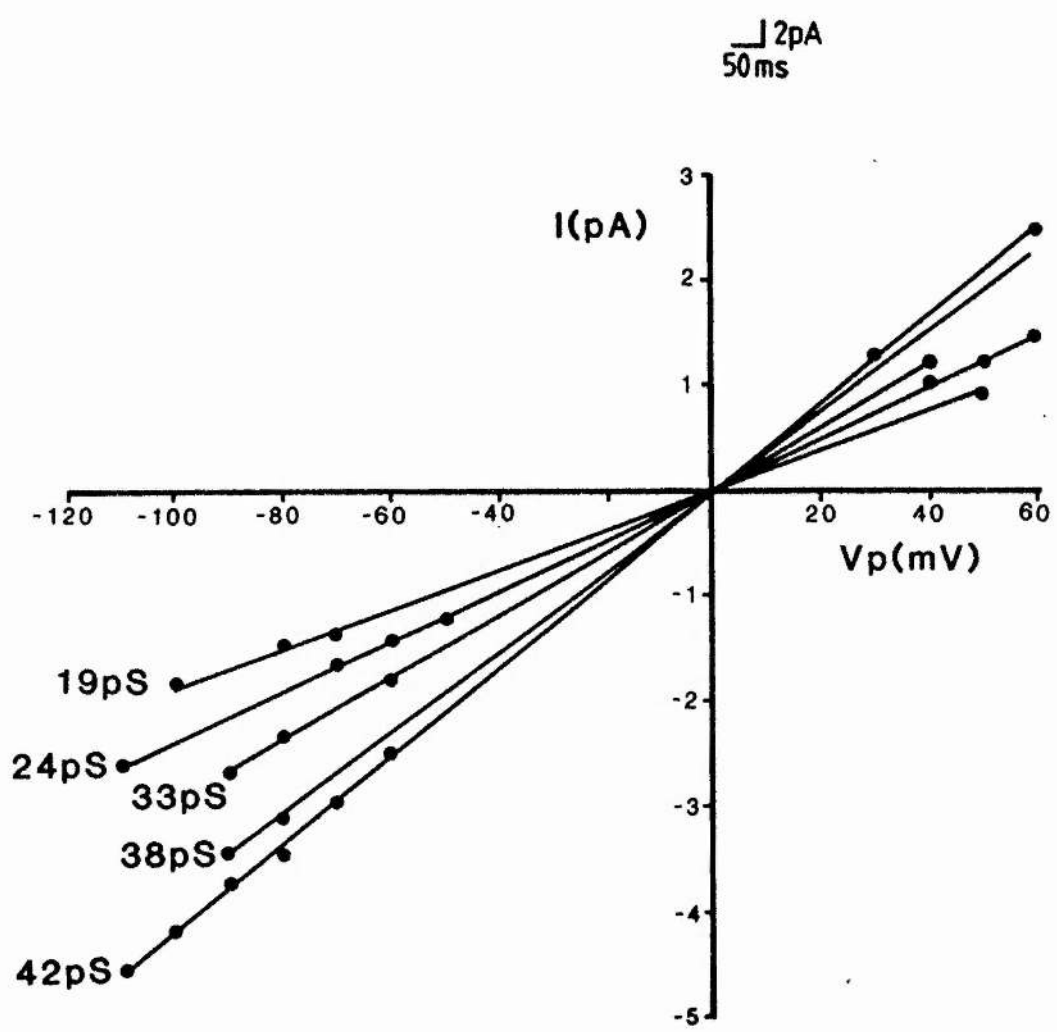
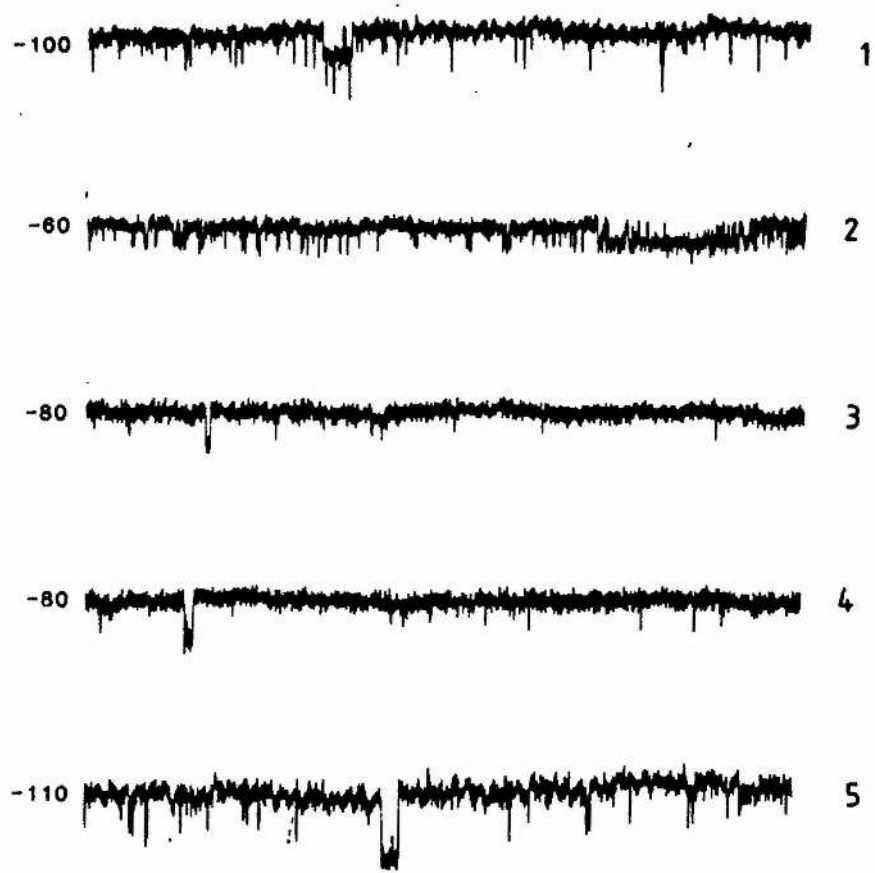
All conductance values quoted were obtained from at least 2 data points and lines fitted by eye were constrained to go through 0mV. However, some variability in the conductance values obtained from the I-V relationship were noted. Such variability was normally in the range of a few pS and was probably due to the temperature of the solutions employed. The conductance values obtained from the I-V plots were noted and compared. Altogether, 156 out of 170 isolated

Figure 3.14

Spontaneous unitary currents in an outside-out patch from a mouse spinal neurone. The patch pipette contained a solution of 140mM TrisCl, while the bath contained a physiological solution of 140mM NaCl. Several different current amplitudes were observed. The number on the left of each trace represents the patch potential in mV.

Traces 1-5 show single-channel currents having slope conductances of 19, 24, 33, 38, and 42pS respectively.

The graph shows the single-channel I-V relationship for the unitary currents described above. The unitary current amplitudes were linearly related to the patch potential. The interpolated reversal potential had a value of 0mV.



outside-out membrane patches showed spontaneous current activity upon excision from the soma. The mean conductances  $\pm$  S.E.M. were obtained and the range (inclusive) from which the mean was obtained is shown below. n represents the number of patches in which the states were present.

(1) $7.17 \pm 0.31\text{pS}$ (n=6)	6-8pS
(2) $12.67 \pm 1.44\text{pS}$ (n=3)	12-15pS
(3) $20.43 \pm 0.43\text{pS}$ (n=33)	16-24pS
(4) $29.26 \pm 0.43\text{pS}$ (n=51)	25-33pS
(5) $37.13 \pm 0.44\text{pS}$ (n=15)	34-40pS
(6) $44.25 \pm 0.63\text{pS}$ (n=24)	41-48pS

The mean conductance values for the larger unitary current events were:

(7) $51.00 \pm 0.35\text{pS}$ (n=15)	49-53pS
(8) $57.11 \pm 0.55\text{pS}$ (n=18)	54-61pS
(9) $69.00 \pm 1.41\text{pS}$ (n=4)	66-70pS
(10) $100.00 \pm 1.04\text{pS}$ (n=3)	98-103pS

All of the above conductance states were observed in symmetrically distributed  $\text{Cl}^-$  solutions and with various different monovalent cations bathing the membrane patch for example  $\text{Na}^+$ ,  $\text{Cs}^+$ ,  $\text{K}^+$  and  $\text{Tris}^+$ .

### 3.4.3 Spontaneous unitary currents in asymmetrical $\text{Cl}^-$ solutions.

In order to validate the hypothesis that the spontaneous unitary currents observed in the outside-out patches were predominantly  $\text{Cl}^-$  conducting, the high  $\text{Cl}^-$  concentration within the patch pipette was replaced with a low  $\text{Cl}^-$  concentration of 30mM ( $a_{\text{Cl}}$  activity 23mM). This low  $\text{Cl}^-$  solution was that used by Hamill et al. in 1983. The bath solution remained a physiological solution containing 140mM NaCl. The calculated  $\text{Cl}^-$  equilibrium potential using the above solutions was again -38mV. Isolated outside-out patches still displayed spontaneous unitary currents. These unitary currents were outward in direction at positive patch potentials and inward at large negative patch potentials. Their I-V relationship was non-linear.

Figure 3. 15 shows examples of spontaneous outward currents in an outside-out patch and their I-V relationship. The outside-out patch described in the figure showed two distinct levels of current amplitude. Their I-V relationship was non linear and was well fit using the Goldman, Hodgkin and Katz (GHK) equation, permeability values of  $7.70 \times 10^{-14}$  and  $10.7 \times 10^{-14} \text{ cm}^3 \text{ s}^{-1}$  which were obtained from the 30pS and 44pS conductance states respectively. Spontaneous current activity was found in 10 out of 11 outside-out patches isolated with these solutions, different conductance states were again

present. In 5 of the longer lasting patches the interpolated reversal potential of the spontaneous events was  $-36 \pm 0.71\text{mV}$ . Similar spontaneous current activity and reversal potential was obtained with a solution containing mainly  $140\text{mM}$  TrisCl replacing the physiological solution in the bath. This reversal potential obtained strongly suggests that these spontaneous events are primarily  $\text{Cl}^-$  conducting.

#### 3.4.4 Transitions between different conductance states

Transitions between various different conductance states could occasionally be observed on certain outside-out patches. Owing to the brief open times of the spontaneous events, careful analysis of the patches had to be performed in order to spot these transitions. Figure 3.16 shows transitions between different conductance levels obtained from various different outside-out patches. As described earlier, openings to large conductance states were relatively rare, however, when they did occur they were usually brief and were sometimes associated with a transition to a sub-conductance level. The figure shows large conductance states ( $>45\text{pS}$ ) and transitions from these states to lower conductance values. Some of these transitions to lower states involved the 30 and 45pS conductance levels.

Figure 3.15

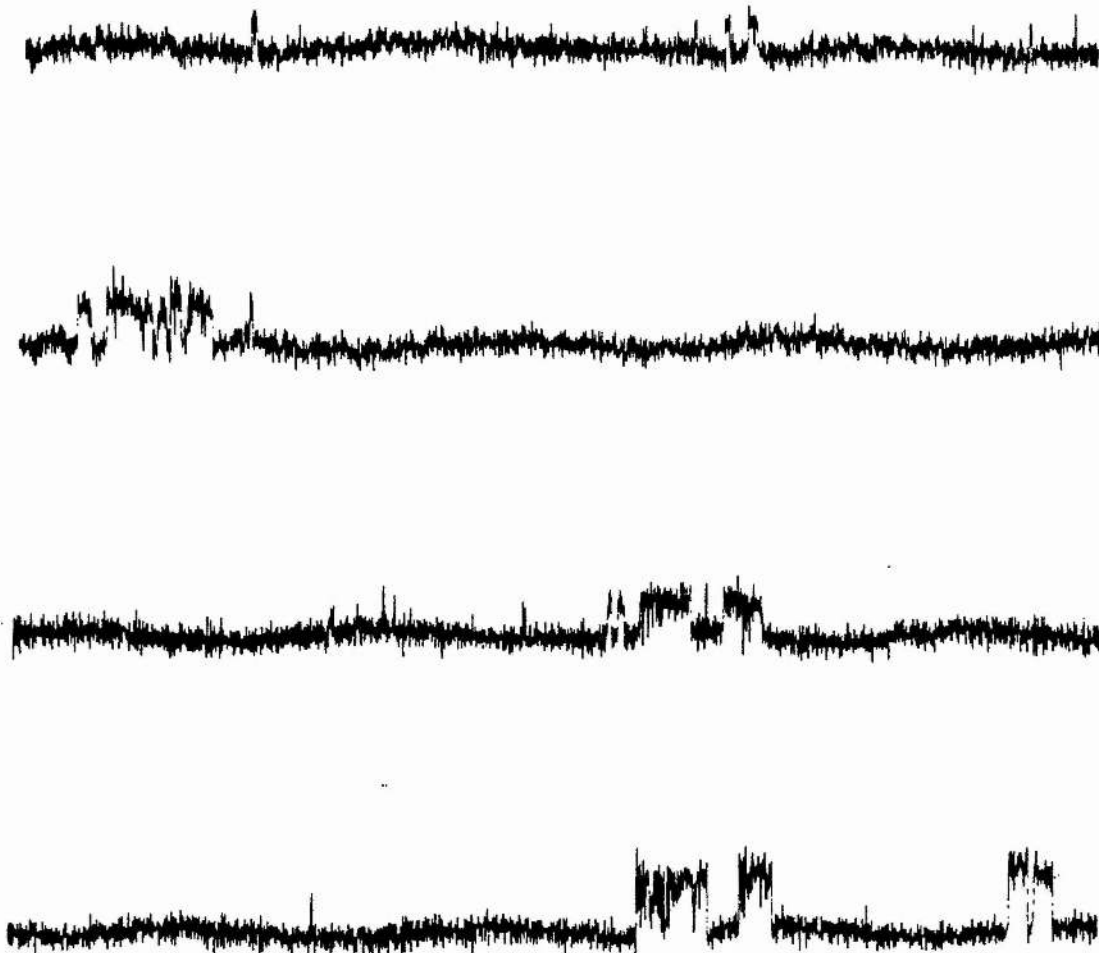
Spontaneous unitary currents observed in an outside-out patch from a mouse spinal neurone at different patch potentials. The patch pipette contained a 25mM CsCl solution, while the bath contained a physiological solution of 140mM NaCl.

The top two traces represent different unitary current amplitudes at a patch potential of +47mV.

The bottom two traces represent unitary currents at a patch potential of +67mV.

Please note unwanted 50Hz in these traces.

The graph shows the relationship between the patch potential ( $V_p$ ) and the single-channel current amplitudes. The lines were drawn using the Goldman-Hodgkin-Katz (GHK) equation, with permeability values of 7.5 and  $10.7 \times 10^{-14} \text{ cm}^3 \text{ s}^{-1}$  and used  $\text{Cl}^-$  activities of 23 and 105mM for the pipette and bath solutions respectively.



2pA  
100ms

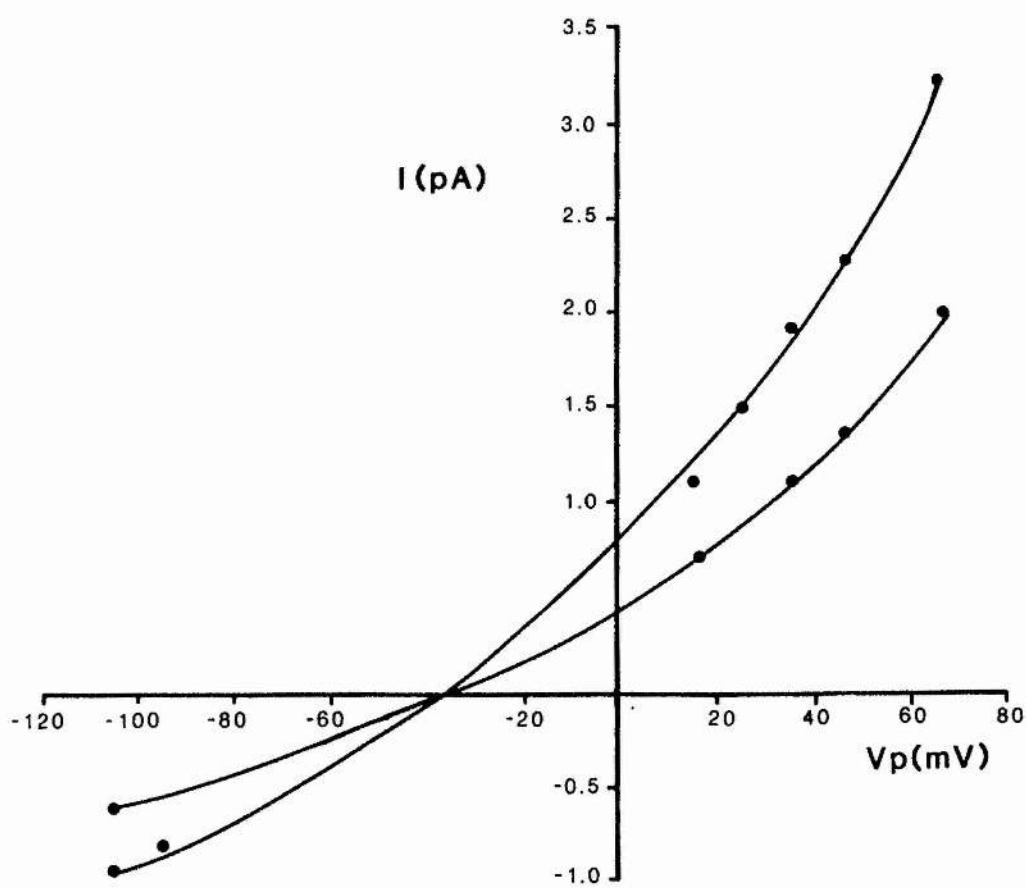




Figure 3.16

Transitions between different conductance states from various outside-out patches obtained from mouse spinal neurones. These patches were all obtained in symmetrical  $\text{Cl}^-$  solutions. The numbers on the left of each trace represent the patch potential in mV.

The top trace shows a transition between a 43pS and 81pS conductance level.

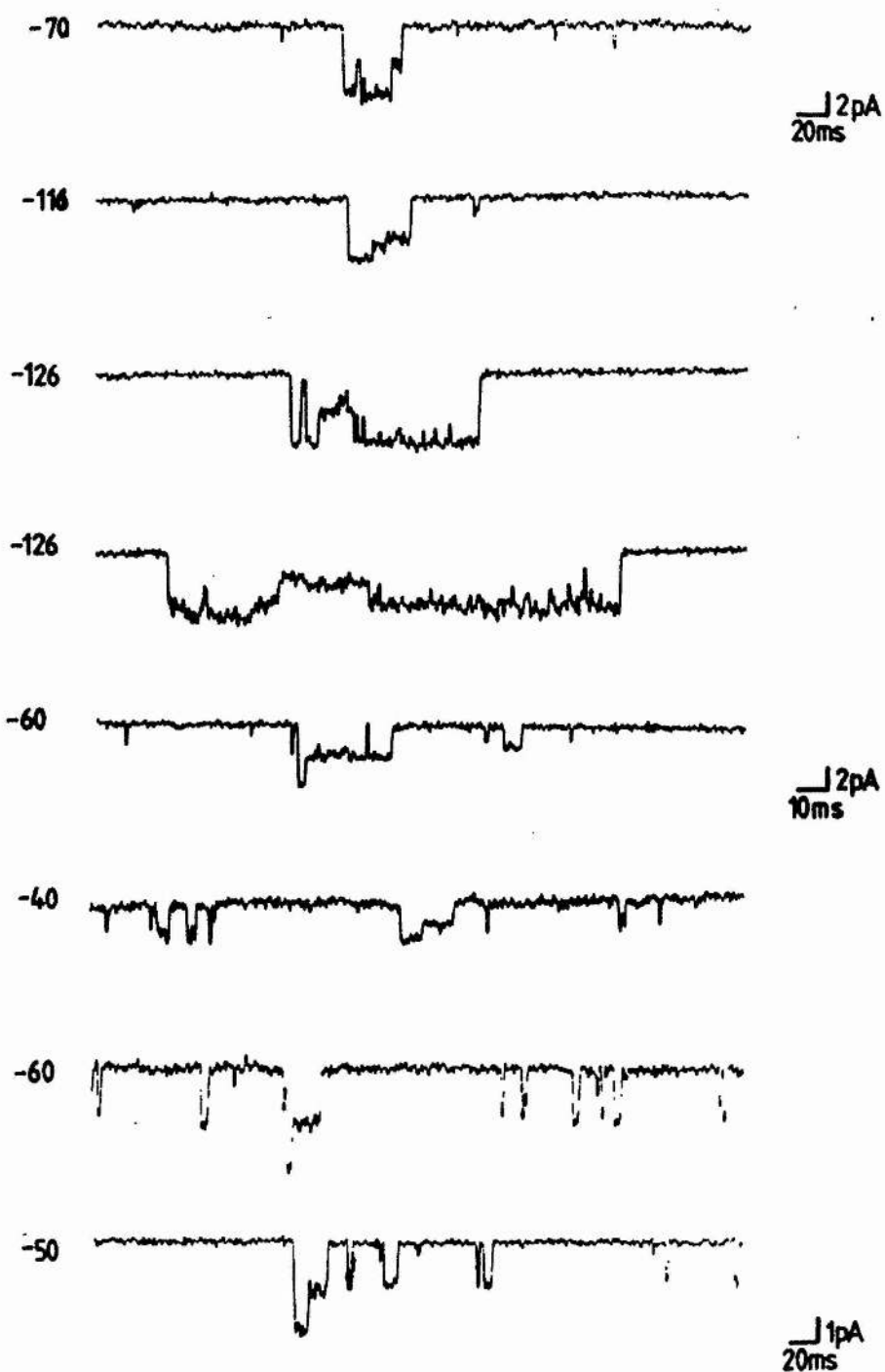
The second trace shows transitions between 3 different levels of 29pS, 33pS and 44pS.

The third trace shows transitions between levels of 17, 26, and 47pS.

The fourth trace shows transitions between levels of 17, 21, 35 and 42pS. The fifth trace shows transitions between a 42pS and 82pS conductance level.

The sixth trace shows transitions between levels of 23pS and 38pS.

The seventh trace shows transitions between a 38pS and 67pS level. The bottom trace shows transitions between a 40pS and a 73pS level.



#### 3.4.5 Effect of bicuculline on spontaneous unitary currents

The nature of these spontaneous events was studied using bicuculline. Outside-out patches displaying spontaneous current activity were exposed to 10 $\mu$ M bicuculline by bath perfusion. Exposure to bicuculline blocked virtually all spontaneous activity in outside-out patches (n=5). Figure 3.17 shows the effect of bath perfusion of bicuculline on the spontaneous activity of an outside-out patch held at -60mV. This particular patch had several different conductance states including a large one of approximately 55pS which was also blocked by bicuculline.

#### 3.5 GABA-ACTIVATED UNITARY CURRENTS IN MOUSE SPINAL NEURONES

From the whole-cell voltage clamp experiments, spinal neurones have been shown to possess a large number of GABA<sub>A</sub> receptors. It is therefore not surprising that outside-out patches have GABA<sub>A</sub> receptors on them. Pressure application of GABA (10-100 $\mu$ M) to outside-out patches evoked unitary currents in all of the patches tested (n=9). Figure 3.18 shows the transient unitary currents activated by GABA in an outside-out patch and the relationship between the patch potential (V<sub>p</sub>) and the main unitary current amplitude activated by GABA. The I-V relationship was linear and the slope revealed a conductance of 29pS. The extrapolated reversal potential was 0mV indicating Cl<sup>-</sup> selective events. This conductance value is similar to that previously observed for the GABA mainstate of mouse spinal neurones (Hamill et al., 1983). The total number of GABA<sub>A</sub> activated channels on outside-out patches varied considerably from patch to patch; some

Figure 3.17

Effect of 10 $\mu$ M bicuculline on the spontaneous current activity in an outside-out patch from a mouse spinal neurone.

Bath perfusion of 10 $\mu$ M bicuculline abolished the activity in this patch which displayed several different conductance states. The patch was held at -60mV, the patch pipette contained a solution of 140mM CsCl and the bath contained a physiological solution of 140mM NaCl.

# CONTROL



# 10μM + BICUCULLINE

PATCH POTENTIAL -60mV



2pA  
100ms

2pA  
100ms

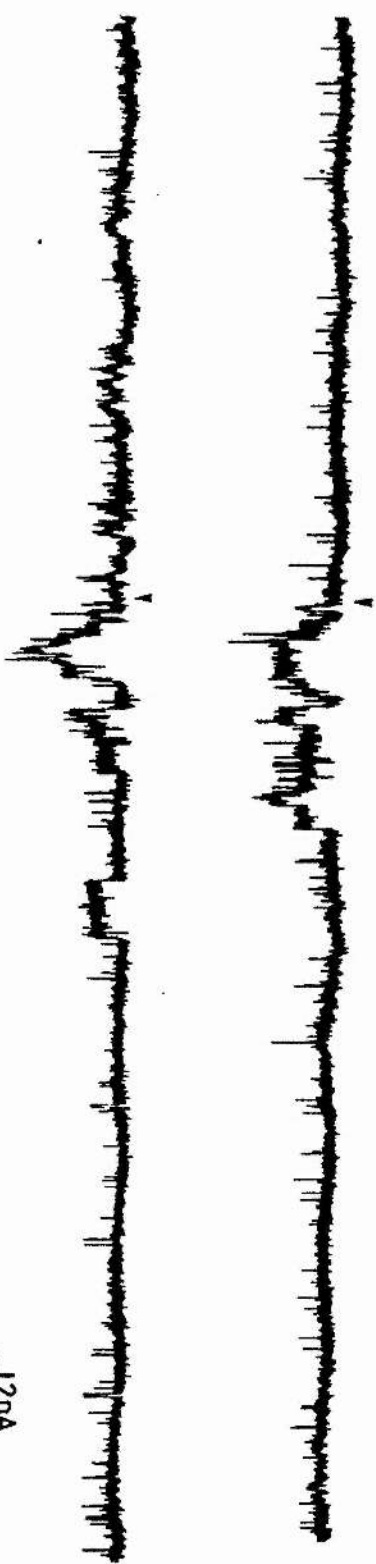


Figure 3.18

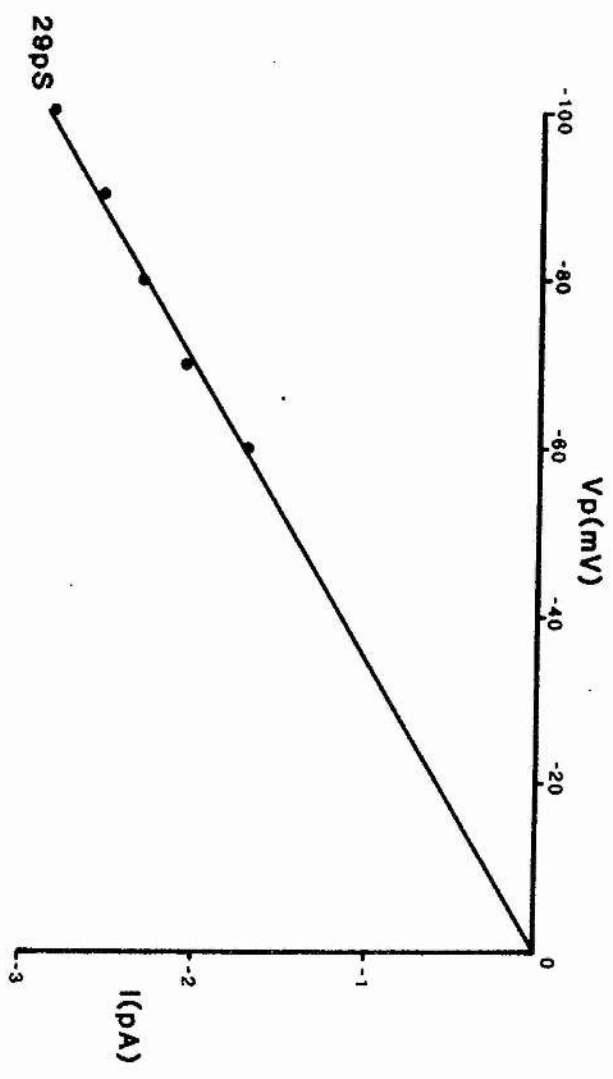
Pressure application of  $10\mu\text{M}$  GABA to an outside-out patch from a mouse spinal neurone. This patch was isolated in symmetrical  $\text{Cl}^-$  solutions with the patch pipette containing a  $140\text{mM}$  KCl solution while the bath contained a physiological solution of  $140\text{mM}$  NaCl.

The patch was held at  $-70\text{mV}$  (top trace) and  $-80\text{mV}$  (bottom trace). GABA was applied for  $130\text{msec}$  (indicated by arrows) and activated inwardly directed unitary currents.

The relation between the patch potential ( $V_p$ ) and the main unitary current amplitude activated by GABA is shown in the graph. The amplitude of the main GABA-activated state was linearly related to the patch potential, and had a slope conductance of approximately  $29\text{pS}$ . The extrapolated reversal potential was  $0\text{mV}$ .



└ 2pA  
50ms



patches had anything up to 10 channels while others had just a few.

Pressure application of GABA ( $10\mu\text{M}$ ) to outside-out patches isolated in asymmetrical  $\text{Cl}^-$  solutions, also evoked transient unitary currents. Figure 3.19 shows the unitary outward currents activated by GABA in 2 different outside-out patches. Here again there was considerable variability in the number of channels present on a patch. The top trace in the figure shows a large number of channels activated by GABA while the bottom trace shows just a few.

### 3.5.1 Multiple conductance states activated by GABA in excised patches

Isolated outside-out patches from mouse spinal neurones showing a low frequency of spontaneous activity were exposed to low concentrations of GABA ( $1-10\mu\text{M}$ ) by bath perfusion. Exposure of the patch to GABA resulted in an increased frequency of opening events and the appearance of bursting activity. Multiple conductance states activated by GABA were observed. Figure 3.20 shows the main conductance states activated by  $2\mu\text{M}$  GABA in an outside-out patch. The dominant conductance state was a  $30\text{pS}$  level but conductances of  $20$  and  $44\text{pS}$  were also activated occasionally. The second part of the figure shows bursting activity of outward unitary currents evoked by GABA in an outside-out patch in asymmetrical  $\text{Cl}^-$  solutions. GABA activated two distinct current levels in this patch.



Figure 3.19

Pressure application of 10 $\mu$ M GABA to outside-out patches obtained from mouse spinal neurones. The patch pipette contained a low Cl<sup>-</sup> solution of 25mM CsCl, while the bath contained a physiological solution of 140mM NaCl.

a: 10 $\mu$ M GABA applied (shown by arrow) to an outside-out patch at a patch potential of +34mV resulted in a large transient outward current.

b: Application of 10 $\mu$ M GABA to another outside-out patch held at +66mV. Application of GABA (shown by arrow) primarily activated unitary currents of approximate amplitude 2pA. This patch also displayed spontaneous current activity prior to the GABA application.

a



10pA  
500ms

b



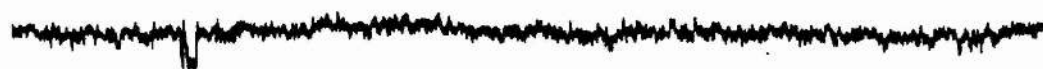
4pA  
50ms

Figure 3.20

Multiple conductance states activated by bath perfusion of  $2\mu\text{M}$  GABA in an outside-out patch from a mouse spinal neurone. The patch pipette contained a solution of  $140\text{mM}$  KCl, while the bath contained a physiological solution of  $140\text{mM}$  NaCl. The patch potential was  $-70\text{mV}$ .

The unitary current in the top trace shows a conductance of  $21\text{pS}$ , while that in the middle trace shows the main GABA conductance state of  $30\text{pS}$ ; finally the bottom trace shows a conductance level of  $45\text{pS}$ . The patch pipette contained a  $140\text{mM}$  KCl solution.

The second part of the figure shows multiple conductance states activated by bath perfusion of  $2\mu\text{M}$  GABA in an outside-out patch from a spinal neurone. The patch pipette contained a low  $\text{Cl}^-$  solution of mainly  $25\text{mM}$  CsCl, while the bath contained a physiological solution of  $140\text{mM}$  NaCl. The patch potential was  $47\text{mV}$  and GABA-activated unitary currents of amplitude  $2.2\text{pA}$  (top trace) and  $1.3\text{pA}$  (bottom trace) at this potential. This patch had previously shown spontaneous activity as described in figure 3.15. The GABA-evoked unitary currents were similar in amplitudes to the spontaneous events but were more frequent and longer in open time duration.



2pA  
100ms



2pA  
100ms

### 3.6 SPONTANEOUS UNITARY CURRENTS FROM RAT DRG NEURONES

#### 3.6.1 Spontaneous unitary currents in symmetrical $\text{Cl}^-$ solutions

Isolated outside-out patches from the somato of DRG neurones also exhibited spontaneous unitary currents. The patch pipette on most occasions had a solution containing mainly 140mM CsCl, while the bath remained a physiological solution containing 140mM NaCl. Various different current amplitudes were observed and again most of the open events were brief, but longer openings were also observed occasionally. I-V relationships for the spontaneous currents were constructed from the longer lasting patches. The number of conductance states present on a particular patch varied along with their activity. An example of the different conductance states present on one of the outside-out patches is shown in figure 3.2 along with their I-V relationship. This patch showed 5 distinct current levels of slope conductance 11, 17, 24, 31 and 36pS. The I-V relationship was linear and the reversal potential was 0mV. The frequency of the different conductance states is indicated by the number of data points in the graph. Large conductance events as revealed from the I-V relationships were also occasionally found in the outside-out patches; these also had a reversal potential of 0mV and a linear I-V relationship.

Figure 3.21

Spontaneous unitary currents in an outside-out patch from a rat DRG neurone. The patch pipette contained a solution of 140mM CsCl and the bath contained a physiological solution of 140mM NaCl.

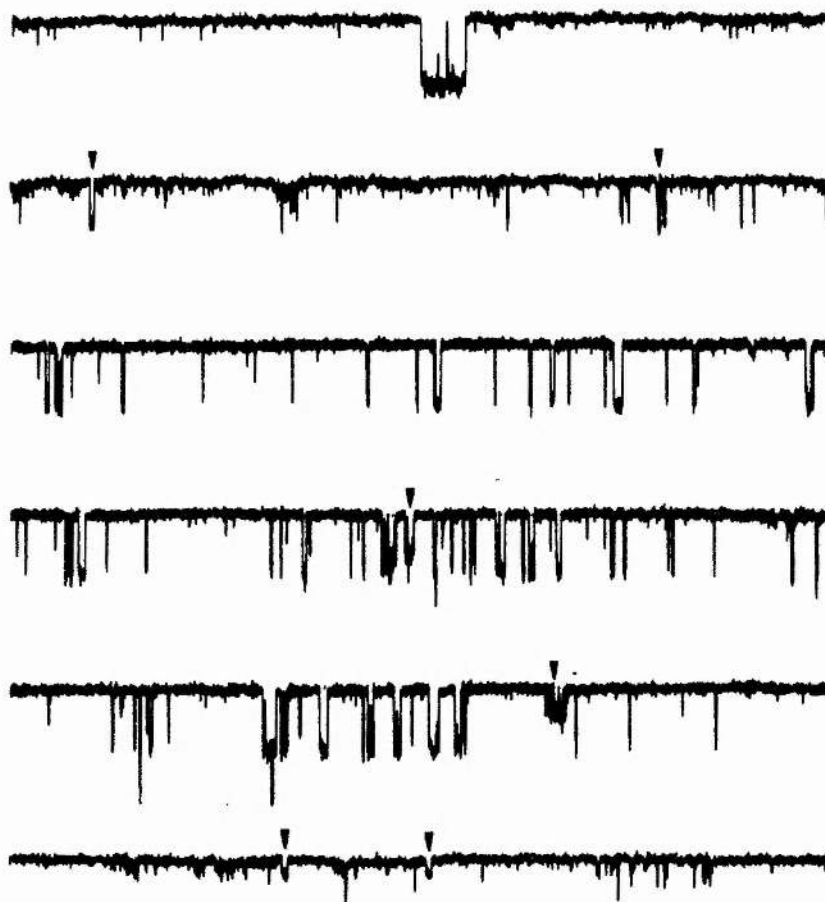
Several different current amplitudes were observed, examples of which are shown in the six traces. The patch was held at -65mV for the first 5 traces and -75mV for the last trace.

The top trace shows a unitary current of conductance 36pS. The arrows in the second trace show channels having a conductance of 24pS.

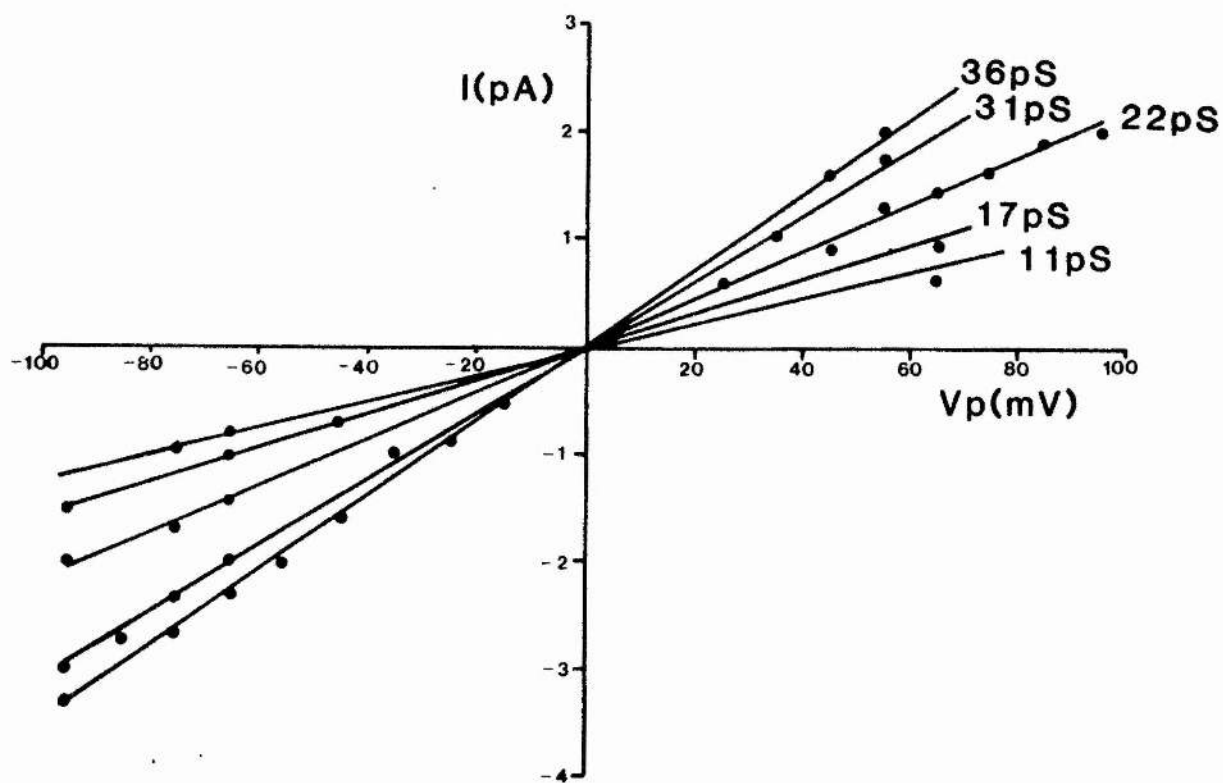
The third trace shows channels of conductance 31pS; the arrow in the fourth trace shows a channel of conductance 17pS, whereas the arrow in the fifth trace shows a channel of conductance 11pS.

An occasional very small conductance channel of 7pS was also observed (bottom trace).

The I-V relationship for the unitary currents described above is shown in the graph. Current amplitudes were linearly related to patch potential ( $V_p$ ) and had an interpolated reversal potential of 0mV. The slope conductances of the most frequently occurring events were 11, 17, 22, 31, and 36pS.



1pA  
100ms



### 3.6.2 Lack of effect of cations on spontaneous unitary currents.

In order to determine the ionic selectivity of these spontaneous currents, different monovalent cations were used in the patch pipette including choline, Tris and potassium. Outside-out patches obtained from rat DRG neurones using these cations still displayed spontaneous unitary currents which had a linear I-V relationship and a reversal potential of 0mV. Figure 3.22 shows examples of spontaneous unitary currents and their I-V relationship in an outside-out patch with a solution containing mainly 140mM cholineCl in the patch pipette. Unitary current amplitudes were still linearly related to the patch potential and had a reversal potential of 0mV. These results suggest that the spontaneous unitary currents were primarily  $\text{Cl}^-$  conducting. Most of the outside-out patches obtained were moved a considerable distance away from the cell from which they were excised and some patches were also perfused with physiological solution at a slow rate of 1-3ml per minute. No conspicuous differences in the activity of the spontaneous currents was observed by either of the above treatments.

All of the conductance states obtained in symmetrically distributed  $\text{Cl}^-$  solutions were pooled and compared. The mean conductance state for the most frequently occurring states was calculated.

(1) $12.17 \pm 0.60\text{pS}$ (n=7)	10-14pS
(2) $17.50 \pm 0.23\text{pS}$ (n=12)	14-19pS
(3) $23.55 \pm 0.41\text{pS}$ (n=18)	21-26pS



(4) $30.00 \pm 0.44\text{pS}$ (n=23)	27-33pS
(5) $36.00 \pm 0.36\text{pS}$ (n=6)	34-38pS
(6) $41.33 \pm 1.00\text{pS}$ (n=9)	39-46pS

Larger conductance states ( $>45\text{pS}$ ) were also occasionally observed like those found in spinal neurones these tended to have very short open times.

(7) $51.13 \pm 0.48\text{pS}$ (n=8)	49-53pS
(8) $59.00 \pm 0.58\text{pS}$ (n=4)	54-60pS
(9) $66.67 \pm 1.23\text{pS}$ (n=6)	63-70pS

### 3.6.3 Transitions between different conductance states

Occasionally transitions between various different conductance states were observed amongst the spontaneous unitary currents in the outside-out patches. Examples of such transitions are shown in figure 3.23. Clear transitions between the 30pS state and other sub- and supra-levels were seen. Some of these were as large as 56pS and as small as 13pS.

Figure 3.22

Spontaneous unitary currents observed in an outside-out patch from a rat DRG neurone. The patch pipette contained a solution of 140mM cholineCl, while the bath was a physiological solution of 140mM NaCl.

At least 3 different conductance states were observed on this patch, the main conductance state was 30pS but smaller states of 19pS (shown by arrow) were also occasionally observed. The graph shows the I-V relationship for these currents, the I-V relationship was linear and had an extrapolated reversal potential of 0mV.

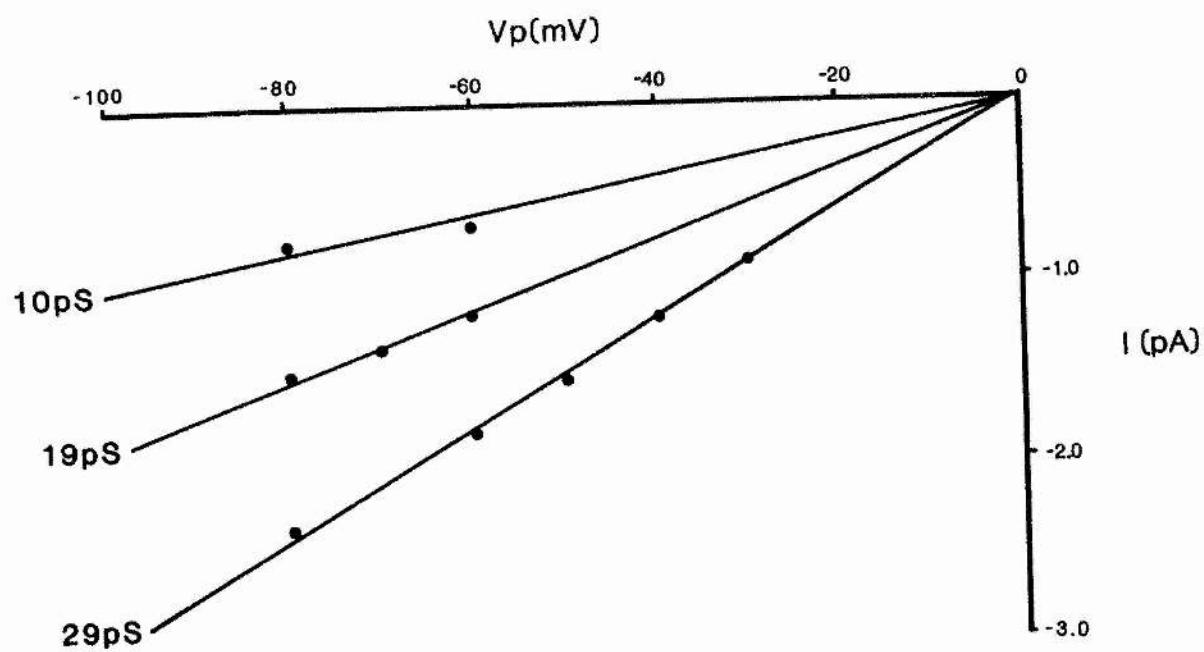


Figure 3.23

Transitions between different conductance states from various outside-out patches obtained from rat DRG neurones. These patches were all obtained in symmetrical  $\text{Cl}^-$  solutions. The numbers on the left of each trace represent the patch potential in mV.

The first trace shows a transition between a 30pS and 17pS conductance level.

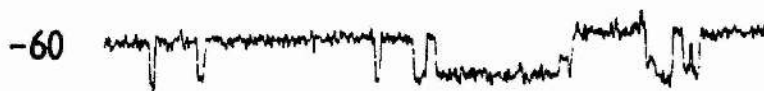
The second trace shows a transition between a 56pS and 31pS conductance level.

The third trace shows a transition between a 13pS and 21pS level; while the fourth trace shows a transition between an 18pS and 41pS level.

The fifth trace shows transitions between a 55pS and a 31pS conductance level.

The sixth trace shows transitions between 18/27pS and 18/13pS.

The last trace shows a transition between levels of 56 and 31pS.



└ 1pA  
20ms

└ 1pA  
10ms

#### 3.6.4 Spontaneous unitary currents in asymmetrical $\text{Cl}^-$ solutions

To support the suggestion that the spontaneous unitary currents were  $\text{Cl}^-$  conducting experiments were performed with the low  $\text{Cl}^-$  (25mM CsCl) solution in the patch pipette. Excised outside-out patches still displayed spontaneous unitary currents, which were outward at positive patch potentials and inward at large negative patch potentials. The I-V relationship of these currents were non-linear. Figure 3.24 shows examples of spontaneous unitary currents at different patch potentials and their I-V relationship. The I-V relationship was non-linear and was fitted well by the constant field equation (GHK) for a  $\text{Cl}^-$  current. Several different conductance states were again found in the outside-out patches. The mean reversal potential for the spontaneous currents was  $-35.71 \pm 1.21\text{mV}$  ( $n=7$ ). In one patch obtained with a solution containing mainly 140mM TrisCl in the bath, similar spontaneous activity and reversal potential was obtained.

#### 3.6.5 Effect of bicuculline on spontaneous unitary currents

The effect of bicuculline on the spontaneous unitary currents was investigated. Bath perfusion of 10 $\mu\text{M}$  bicuculline was capable of abolishing the spontaneous unitary currents in 4 patches. Figure 3.25 shows an example of this abolishment of the spontaneous unitary currents in an outside-out patch by bicuculline.

Figure 3.24

Spontaneous unitary currents observed in an outside-out patch from a rat DRG neurone at different patch potentials.

The patch pipette contained a 25mM CsCl solution, while the bath contained a physiological solution of 140mM NaCl. Different current amplitudes were observed. The numbers on the left represent the patch potential in mV.

At -74mV small inwardly directed currents were observed, at -34mV the patch showed little if any current activity. At +06mV patch potential small outward channels were viewed, and these increased in amplitude at a patch potential of +16mV.

The relationship between the patch potential ( $V_p$ ) and the main unitary current amplitude (I-V relation) is shown in the graph. The graph was drawn using the Goldmann Hodgkin and Katz equation for a  $\text{Cl}^-$  current, using a permeability value of  $5.65 \times 10^{-14} \text{ cm}^3 \text{ s}^{-1}$  and  $\text{Cl}^-$  activities of 23 and 105mM for the pipette and bath solutions respectively.



└ 1pA  
50ms

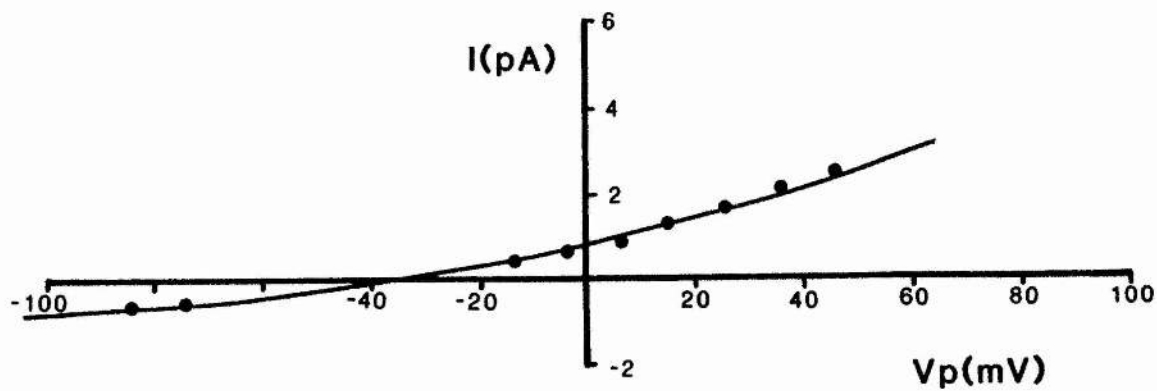
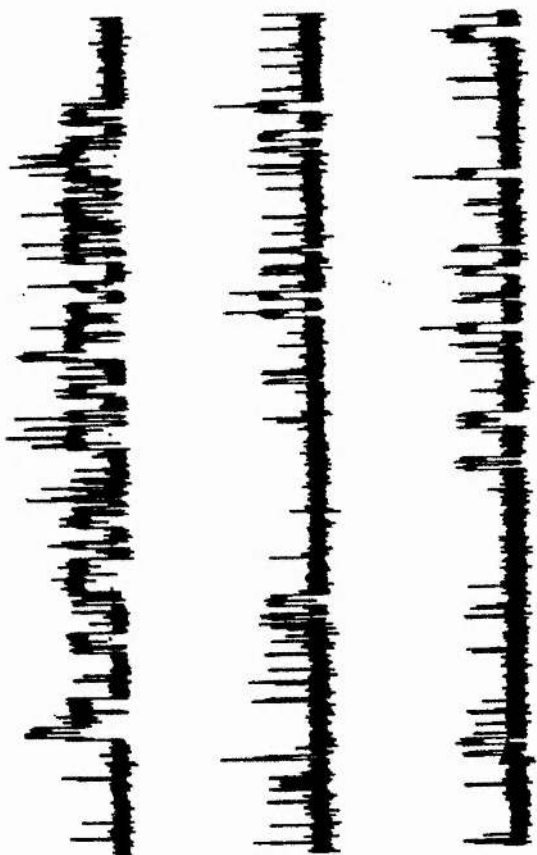




Figure 3.25

The effect of bath perfusing  $10\mu\text{M}$  bicuculline on the spontaneous current activity in an outside-out patch from a rat DRG neurone. This patch displayed different unitary current amplitudes at the patch potential of  $-70\text{mV}$ .

CONTROL



10  $\mu$ M BICUCULLINE



1 pA  
100 ms

### 3.7 GABA-ACTIVATED UNITARY CURRENTS IN RAT DRG NEURONES

The effect of pressure application of GABA (10-50 $\mu$ M) to isolated outside-out patches was investigated. As with the spinal neurones application of GABA-activated unitary currents, the number of which varied from patch to patch. GABA-activated unitary currents, in six outside out patches tested. Figure 3.26 shows an example of the unitary currents activated by GABA in an outside-out patch. The unitary current amplitudes were linearly related to the patch potential and these currents had an extrapolated reversal potential of 0mV. The slope conductance of the main GABA activated state was 28pS. Apart from this main state other conductance levels were also activated by GABA. Figure 3.27 shows conductance levels of 13, 20 and 30pS activated by pressure application of 30 $\mu$ M GABA to an outside-out patch.

### 3.8 EFFECT OF DIAZEPAM ON GABA-EVOKED WHOLE-CELL CURRENTS

The effect of different concentrations of the benzodiazepine agonist diazepam was investigated on submaximal whole-cell currents in spinal neurones. The submaximal GABA-evoked currents were achieved in one of two ways, by either reducing the duration of the ejection pulse or by slightly withdrawing the pressure pipette from the cell under study. Skerritt and MacDonald (1984a) reported potent enhancing actions of benzodiazepine agonists (diazepam, clonazepam, and nitrazepam) on GABA responses in mouse spinal neurones. These benzodiazepine agonists were shown to increase GABA responses in the low nM range.

Figure 3.26.

Pressure application of 30 $\mu$ M GABA to an outside-out patch from a rat DRG neurone at two different patch potentials of -50 and -60mV.

Application of GABA (shown by arrows) evoked transient inward unitary currents which increased in amplitude with patch hyperpolarization. The graph shows the I-V relationship of the main conductance state activated by GABA. The extrapolated reversal potential was 0mV and the slope revealed a conductance of 28pS. The patch pipette contained a solution of 140mM KCl, while the bath contained a physiological solution of 140mM NaCl.

PATCH POTENTIAL -60mV



PATCH POTENTIAL -60mV

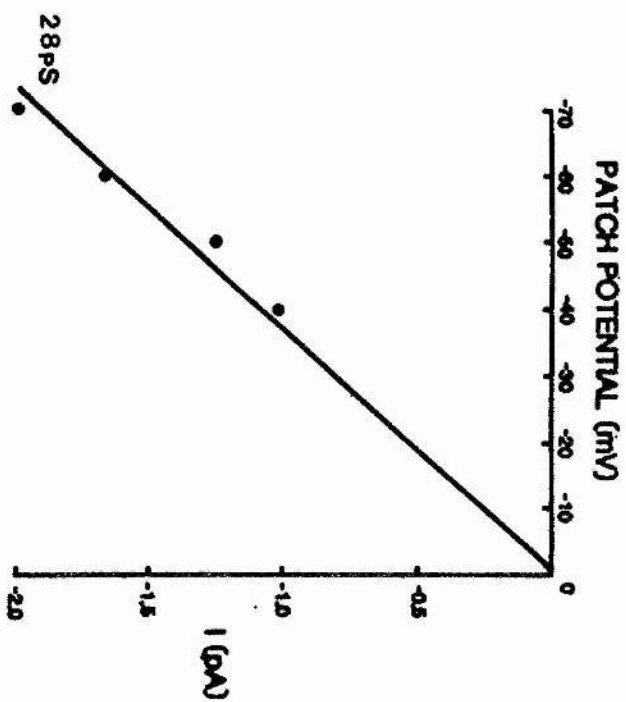
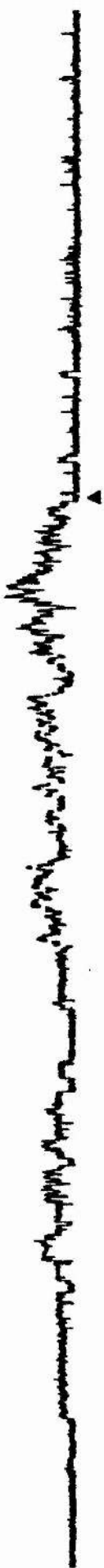


Figure 3.27

Multiple conductance states activated by pressure application of 30 $\mu$ M GABA to an outside-out patch from a rat DRG neurone.

The top trace shows the patch held at -70mV, the arrow shows a conductance of approximately 13pS, while the larger current levels correspond to a conductance of 30pS.

The bottom trace shows the patch held at -60mV, the arrow shows a conductance level of 20pS; the other current levels show the main conductance level of 30pS.

The patch pipette contained a 140mM TrisCl solution, while the bath contained a physiological solution of 140mM NaCl.



└ 2pA  
100ms

A similar concentration of diazepam (10nM) was tested on the spinal neurones to see whether similar results would be obtained. Figure 3.28 shows the enhancing action of 10nM diazepam on GABA-evoked whole-cell currents. Only a small proportion of the spinal neurones responded to 10nM diazepam. Infact only 2 out of 11 cells tested responded with a reversible increase (approx 50%) in the size of the GABA-evoked current. Higher concentrations of diazepam (1 and 10 $\mu$ M) were also tested on spinal neurones. Figure 3.29 shows the effect of 1 $\mu$ M diazepam on GABA-evoked whole-cell currents. 1 $\mu$ M diazepam in 4 cells tested caused a mean increase of the GABA current of  $48.50 \pm 11.44\%$ . The effect of 10 $\mu$ M diazepam on the GABA-evoked whole-cell currents is shown in figure 3.30. The increase in the amplitude of the GABA currents caused by 10 $\mu$ M diazepam was hard to reverse on many cells even after prolonged washing. The mean increase in the size of the current was  $41.88 \pm 5.09\%$  (n=9). Some of the cells (n=4) showed no consistent response to diazepam (1 and 10 $\mu$ M).



Figure 3.28

Effect of 10nM diazepam on 10 $\mu$ M GABA-evoked whole-cell currents in a mouse spinal neurone.

The top trace shows the control GABA currents upon bath perfusion with 10nM diazepam the current was increased (middle trace). This effect was fully reversed on washing (bottom trace). The cell was held at -60mV and the patch pipette contained solution of 140mM CsCl. The bath contained a physiological solution of 140mM NaCl.

CONTROL



10nM DIAZEPAM



WASH



└─ 400pA  
10s

Figure 3.29

Effect of  $1\mu\text{M}$  diazepam on  $20\mu\text{M}$  GABA-evoked whole-cell currents in a mouse spinal neurone.  $1\mu\text{M}$  diazepam reversibly enhanced the current. The holding potential was  $-60\text{mV}$  and the patch pipette contained a  $140\text{mM}$  CsCl solution. The bath contained a physiological solution of  $140\text{mM}$  NaCl.

CONTROL



1 $\mu$ M DIAZEPAM



WASH

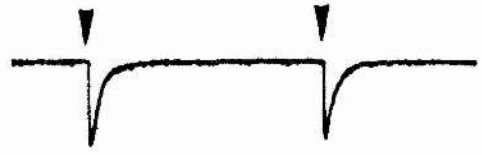


└─ 400pA  
10s

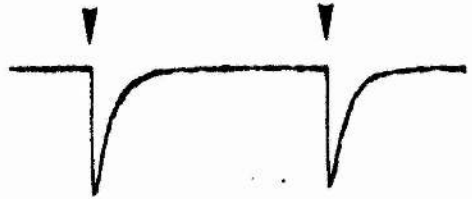
Figure 3.30

Effect of  $10\mu\text{M}$  diazepam on  $20\mu\text{M}$  GABA-evoked whole-cell currents in a mouse spinal neurone.  $10\mu\text{M}$  diazepam enhanced the size of the currents (middle trace). This effect of diazepam was reversed in the wash (bottom trace). The holding potential was  $-60\text{mV}$  and the patch pipette contained a solution of  $140\text{mM}$  CsCl. The bath was a physiological solution containing  $140\text{mM}$  NaCl.

CONTROL



10 $\mu$ M DIAZEPAM



WASH



└─┘ 400pA  
10s

### 3.9 STEROID ACTION ON THE GABA<sub>A</sub> RECEPTOR (WHOLE-CELL STUDIES)

#### 3.9.1 Potentiation of the GABA current by alphaxalone

Alphaxalone, a steroid anaesthetic has been shown to potentiate GABA responses recorded extracellularly from slices of the rat cuneate nucleus (Harrison and Simmonds, 1984). More recently alphaxalone at low concentrations (30nM -1µM) was observed to potentiate the chloride current activated by GABA in isolated bovine adrenal medullary cells and also at higher doses to directly activate the GABA receptors of these cells (Cottrell, Lambert and Peters, 1986).

The effects of alphaxalone on the GABA<sub>A</sub> receptor of mammalian neurones was investigated. GABA-evoked currents in mouse spinal and rat DRG neurones were reversibly potentiated by alphaxalone. Figure 3.31 shows the reversible potentiation of GABA-evoked inward currents upon exposure to 300nM alphaxalone in a spinal neurone. Alphaxalone at 300nM in the medium reversibly increased submaximal whole-cell currents evoked by GABA by  $93 \pm 8.98\%$  (n=6). GABA-evoked whole-cell currents in DRG neurones were also potentiated by alphaxalone. Figure 3.32 shows the effect of 600nM alphaxalone on the inward GABA current of a rat DRG neurone. Alphaxalone at 600nM in the medium reversibly potentiated whole-cell currents evoked by GABA by  $63.75 \pm 6.14\%$  (n=4).

Figure 3.31

Effect of 300nM alphaxalone on inward currents evoked by local application of 10 $\mu$ M GABA to a mouse spinal neurone.

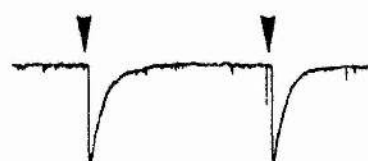
The top trace shows control GABA currents. The middle trace shows the increase in the size of the currents in the presence of 300nM alphaxalone. This effect was reversible in the washout (bottom trace). The holding potential was -70mV, the patch pipette contained a solution of 140mM KCl and the bath contained a physiological solution of 140mM NaCl.



CONTROL



300nM ALPHAXALONE



WASH



200pA  
10s

Figure 3.32

Effect of 600nM alphaxalone on 30 $\mu$ M GABA-activated inward currents in a rat DRG neurone.

The top trace shows the control GABA current. The middle trace shows the increase in the size of the current in the presence of 600nM alphaxalone. The bottom trace shows the reversibility of this effect upon washout. The patch pipette contained a solution of 140mM CsCl, the bath contained a physiological solution of 140mM NaCl. The holding potential was -60mV.

CONTROL



200pA  
2s

600nM ALPHAXALONE



WASH



### 3.9.2 Direct agonist action of alphaxalone on the GABA<sub>A</sub> receptor

Bath perfusion of alphaxalone, at higher concentrations ( $>1\mu\text{M}$ ), caused a larger initial increase in the amplitude of the GABA currents, but there also followed an increase in the baseline current noise. This indicated a direct action of alphaxalone on the membrane. The direct action of alphaxalone was studied using pressure application. The GABA in the pressure pipette was replaced with alphaxalone at concentrations between 10-50 $\mu\text{M}$ .

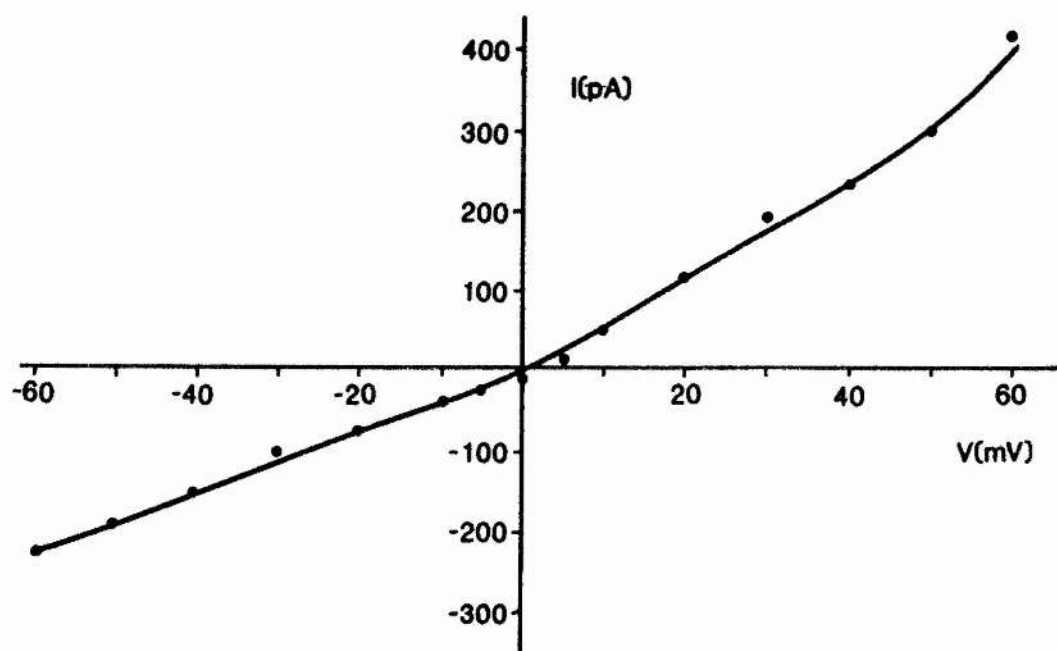
Alphaxalone (10-50 $\mu\text{M}$ ) applied to spinal neurones by pressure ejection evoked transient membrane currents. These currents were outward in sign at positive holding potentials and inward at negative holding potentials and negligible at 0mV. Figure 3.33 shows currents evoked by alphaxalone in a spinal neurone at different holding potentials and the relationship between the holding potential and the size and sign of the currents evoked by alphaxalone. In a few experiments an intracellular solution containing mainly 140mM TrisCl was used in the patch pipette. Similar results were obtained as regards to the currents evoked and the reversal potential obtained. In 10 cells studied the reversal potential of the response was  $-2.25 \pm 0.95\text{mV}$  which was very similar to that obtained for GABA  $-3.60 \pm 0.89\text{mV}$  (n=14) under similar conditions. From the value of the reversal potential obtained, the currents evoked by alphaxalone probably involved  $\text{Cl}^-$ .

Figure 3.33

Direct agonist action of alphaxalone on the GABA<sub>A</sub> receptor of a mouse spinal neurone.

Local application of 50 $\mu$ M alphaxalone alone evoked a membrane current which was inward at a holding potential of -30mV, outward at +50mV and very small at 0mV. Note the different gain of the recording at a  $V_h$  of 0mV. The arrows show where alphaxalone was applied.

The graph shows the relationship between the holding potential ( $V_h$ ) and the size and sign of the currents evoked by local application of 50 $\mu$ M alphaxalone. The patch pipette contained a solution of 140mM CsCl and the bath contained a physiological solution of 140mM NaCl.



### 3.9.3 Effect of bicuculline and phenobarbitone on alphaxalone currents

Experiments were then conducted to see whether these alphaxalone evoked currents could be pharmacologically modulated by drugs known to affect GABA<sub>A</sub> responses. Bicuculline was capable of reversibly suppressing the alphaxalone-evoked current in a manner similar to that observed for GABA responses. Figure 3.34 shows the antagonistic action of bicuculline on the inward current evoked by 50 $\mu$ M alphaxalone. The alphaxalone-evoked current was reversibly suppressed by  $86.5 \pm 4.95\%$  (n=4) when exposed to physiological solution containing 10 $\mu$ M bicuculline.

The effect of 500 $\mu$ M phenobarbitone on the alphaxalone-evoked current was also studied. Phenobarbitone caused a remarkably large increase in the alphaxalone-evoked current. Figure 3.35 shows the reversible potentiation of the alphaxalone-evoked outward current by phenobarbitone. Phenobarbitone at 500 $\mu$ M in the medium reversibly potentiated submaximal alphaxalone-evoked currents by  $648 \pm 38.86\%$  (n=5). Diazepam at 1 $\mu$ M in the bathing solution also reversibly potentiated alphaxalone-evoked currents (data not shown).

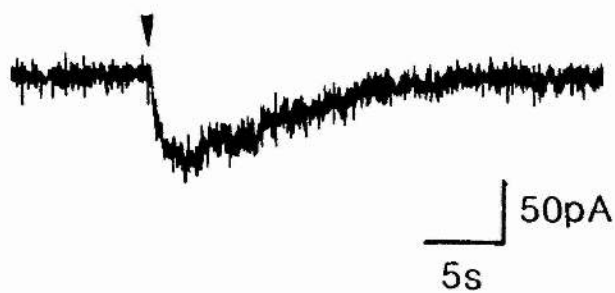
Figure 3.34

Effect of  $10\mu\text{M}$  bicuculline on the  $50\mu\text{M}$  alphaxalone-evoked current in a mouse spinal neurone.

The top trace shows the control current evoked by alphaxalone. In the presence of  $10\mu\text{M}$  bicuculline this current is suppressed (middle trace). This effect is reversed upon washout (bottom trace). The arrows indicate where alphaxalone was applied and the holding potential was  $-60\text{mV}$ . The patch pipette contained a solution of  $140\text{mM}$  CsCl, and the bath contained a physiological solution of  $140\text{mM}$  NaCl.



CONTROL



10 $\mu$ M BICUCULLINE



WASH

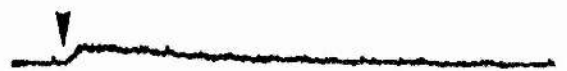


Figure 3.35

Effect of 500 $\mu$ M phenobarbitone on the outward current evoked by 50 $\mu$ M alphaxalone in a mouse spinal neurone.

The top trace shows the control outward current. The middle trace shows the marked enhancement in this current with 500 $\mu$ M phenobarbitone in the bathing solution. This effect was reversed upon washout (bottom trace). The arrows indicate where alphaxalone was applied. The patch pipette contained a solution of 140mM CsCl and the bath contained a physiological solution of 140mM NaCl. The holding potential was +30mV.

CONTROL

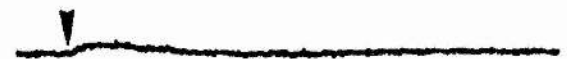


200pA  
4s

500 $\mu$ M PHENOBARBITONE



WASH



### 3.10 EFFECT OF ENDOGENOUS STEROIDS

#### 3.10.1 Potentiation of GABA-evoked currents by pregnanolone

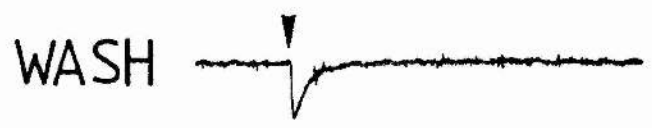
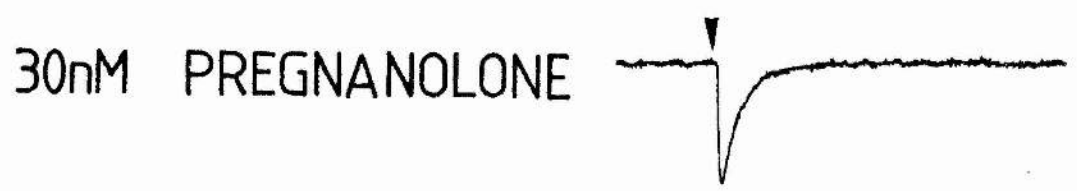
Alphaxalone is structurally very similar to progesterone and its metabolites. The reduced metabolite of progesterone  $5\beta$ -pregnane- $3\alpha$ -ol-20-one (pregnanolone) has been shown to potentiate GABA-evoked currents in bovine chromaffin cells (Callachan et al., 1986). The action of pregnanolone ( $5\beta$ -pregnane- $3\alpha$ -ol-20-one) on GABA-evoked whole-cell currents in spinal neurones was investigated. Pregnanolone reversibly potentiated GABA-evoked whole-cell currents in mouse spinal neurones at very low doses. Concentrations as low as 30nM had a significant potentiating action on the GABA currents.

Figure 3.36 shows the reversible potentiation of GABA-evoked inward currents by 30nM pregnanolone in the bathing solution. In 5 cells exposed to 30nM pregnanolone, the mean increase in the size of the currents was  $78.4 \pm 18.84\%$ . Higher concentrations of pregnanolone caused larger increases in the amplitude of the GABA currents. Figure 3.37 shows the effect of 60nM pregnanolone on the GABA-evoked inward current in a spinal neurone. In 6 cells studied, the mean increase in the size of the GABA currents was  $241.5 \pm 62.52\%$ . The action of 100nM pregnanolone was also studied in 3 cells, the mean increase in the GABA current was  $886.7 \pm 147.62\%$ . The dose dependency of the increase in the GABA current is shown graphically in figure 3.38 where the % increase in the amplitude of the GABA-evoked currents is plotted against the concentration of pregnanolone in the bathing medium.

Figure 3.36

Effect of 30nM pregnanolone (5 $\alpha$ -pregnane-3 $\alpha$ -ol-20-one) on inward currents evoked by 20 $\mu$ M GABA in a mouse spinal neurone. Local application of GABA is indicated by the arrows.

The GABA-evoked current was potentiated in the presence of 30nM pregnanolone (middle trace). This potentiation was reversed in the wash (bottom trace). The patch pipette contained a solution of 140mM KCl and the bath was a physiological solution of 140mM NaCl. The holding potential was -70mV.



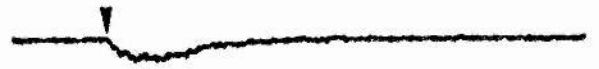
1nA  
5s

Figure 3.37

Effect of 60nM pregnanolone (5 $\beta$ -pregnane-3 $\alpha$ -ol-20-one) on 100 $\mu$ M GABA-evoked currents in a mouse spinal neurone.

The top trace shows control GABA-evoked inward currents. The middle trace shows the increase in the size and duration of the current upon bath perfusion with 60nM pregnanolone. This effect was reversed in the washout (bottom trace). A solution containing 140mM CsCl was used in the patch pipette, while the bath contained a physiological solution of 140mM NaCl. The cell was held at -50mV.

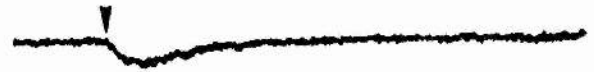
CONTROL



60nM PREGNANOLONE



WASH



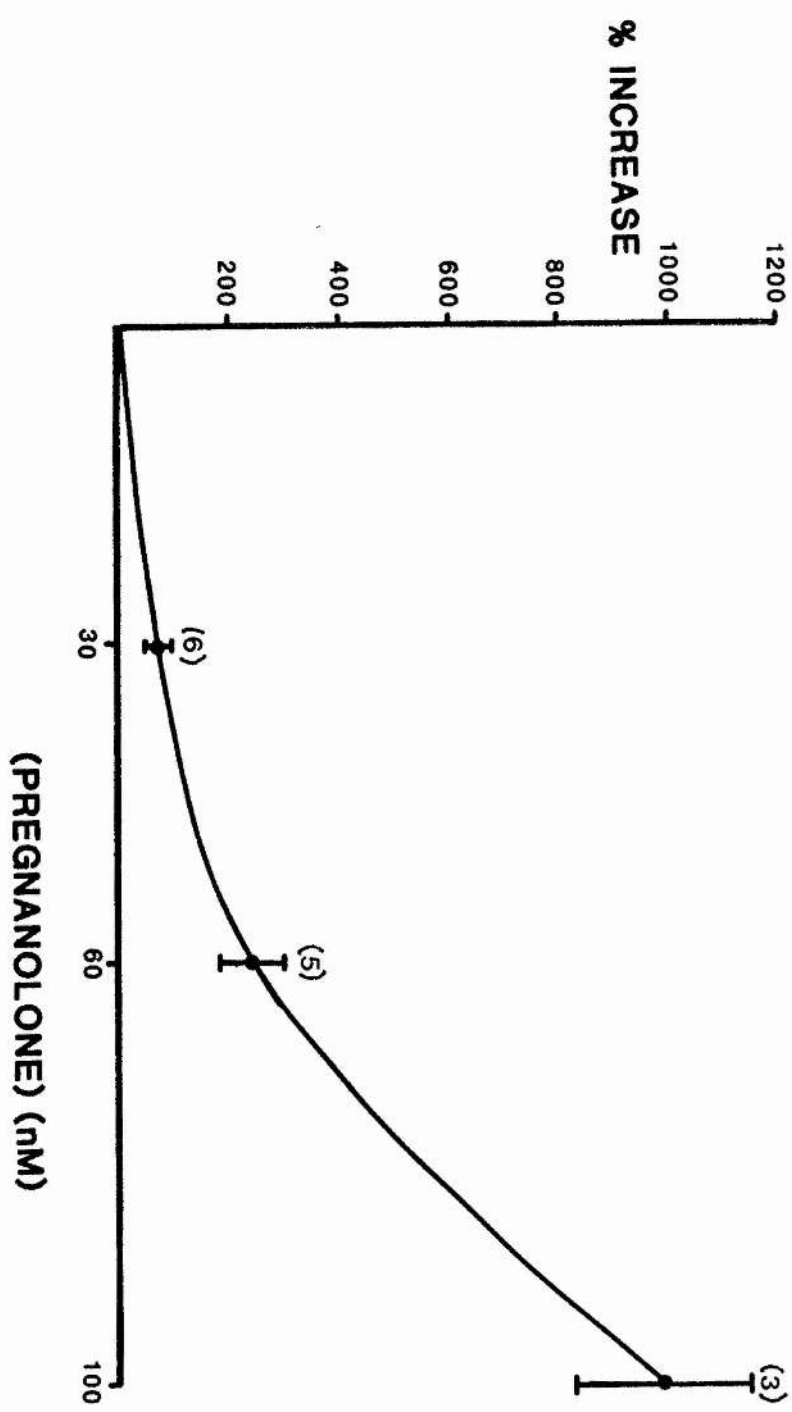
250pA  
2s



Figure 3.38

The dose dependency of the potentiation of GABA-evoked currents by pregnanolone is shown in the graph.

The percentage increase in the amplitude of the GABA-evoked currents is plotted against the concentration of pregnanolone in the bathing medium. The error bars represent the standard error of the mean (S.E.M), and the number within the brackets represent the number of cells tested.



### 3.10.2 Weak direct agonist action of pregnanolone

A direct agonist action of pregnanolone on the GABA<sub>A</sub> receptor was studied. Pregnanolone at a concentration of 10 $\mu$ M in the pressure pipette evoked small (<100pA) membrane currents. In order to evoke sizeable currents larger than the baseline noise, large ejection durations were used along with high (>15 p.s.i.) pressures. The currents produced by pressure application of pregnanolone tended to have long durations (>10s). Figure 3.39 shows membrane currents evoked by pregnanolone at different holding potentials in a spinal neurone. The reversal of the response occurred at 0mV, indicating a Cl<sup>-</sup> selective response; in 4 cells the mean reversal potential was -2.0  $\pm$  1.47mV. Pregnanolone at 100nM in the pressure pipette did not evoke membrane currents in 2 spinal neurones tested.

## 3.11 UNITARY CURRENT STUDIES

### 3.11.1 Mechanism of action of pregnanolone potentiation

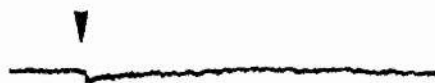
The mechanism of action of the potentiation of GABA currents by pregnanolone was investigated at the single-channel level using outside-out patches from spinal neurones. GABA-activated unitary currents were evoked in outside-out patches by bath perfusion of 1 $\mu$ M GABA, the same patch was then subsequently exposed to 1 $\mu$ M GABA and 200/300nM pregnanolone. A clear difference in the unitary current activity was found, long bursts of channel activity was seen in the

Figure 3.39

Weak direct agonist action of 10 $\mu$ M pregnanolone (5 $\beta$ -pregnane-3 $\alpha$ -ol-20-one) on a mouse spinal neurone held in voltage clamp conditions. The arrows show the effect of pressure ejection of pregnanolone at three different holding potentials. The currents produced are small and are inward at negative holding potential ( $V_h$  -20mV) and outward at positive holding potential (+10mV) and negligible at a holding potential of 0mV.

The relationship between the holding potential and the size and sign of the currents evoked by local application of 10 $\mu$ M pregnanolone is shown in the graph. The patch pipette contained a solution of 140mM CsCl, and the bath contained a physiological solution of 140mM NaCl. The pressure employed was 19 p.s.i. and the ejection duration was 230msecs.

$V_h -20\text{mV}$



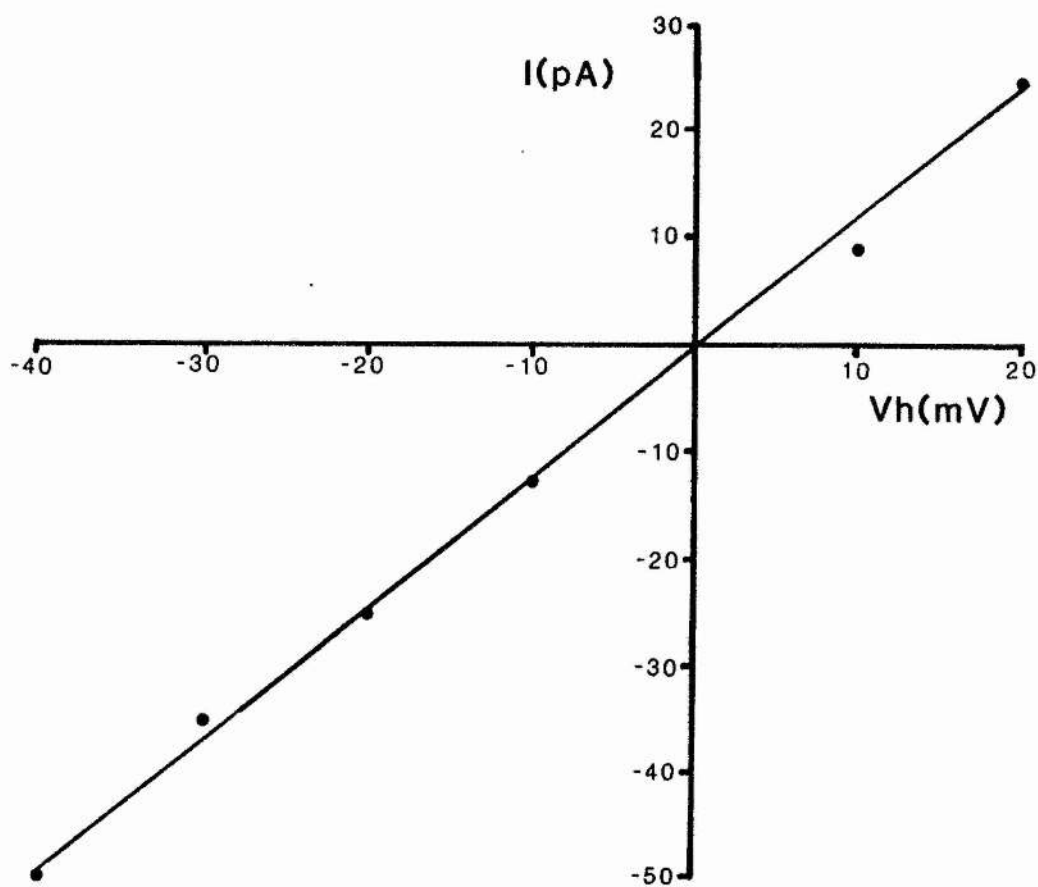
$V_h 0\text{mV}$



$V_h +10\text{mV}$



└ 100pA  
10s



presence of the steroid. Figure 3.40 shows traces of GABA-activated unitary currents in the presence and absence of 200nM pregnanolone (5 $\beta$ -pregnane-3 $\alpha$ -ol-20-one). Kinetic analysis of open and closed time durations were performed of the GABA-activated unitary currents in the presence and absence of pregnanolone (200/300nM) for 3 outside-out patches. Kinetic analysis of the single-channel currents was done with an IBM PC AT computer and software (PAT 3). The single-channel records were filtered at 1kHz and digitized at 100 $\mu$ s per point with an analogue to digital (A/D) converter so that opening and closing transitions could be detected. A 50% threshold level between the closed and main open state (30pS) was used to detect open events. Openings of the channel to sub- and supra-conductance levels were edited out of the final record along with multiple openings. A burst was defined as a group of openings separated by channel closures lasting less than 5ms. The 3 outside-out patches obtained varied somewhat in the number of GABA-activated channels present; patch 1 had only one or two channels on it, while the other two patches probably had more than this as was concluded from the occasional multiple opening. The variability in the kinetic parameters shown in Table 3.1 probably represents the differences in the number of channels present on these patches. Various different kinetic parameters were obtained from the unitary currents (see Table 3.1), the mean value of each parameter is shown in the presence and absence of steroid for each of the 3 outside-out patches.

From inspection of the GABA-activated unitary currents and the table, the main kinetic parameter affected was the mean burst length. An increase in the channel open probability was also noted. The mean closed time was not greatly affected by the steroid and small

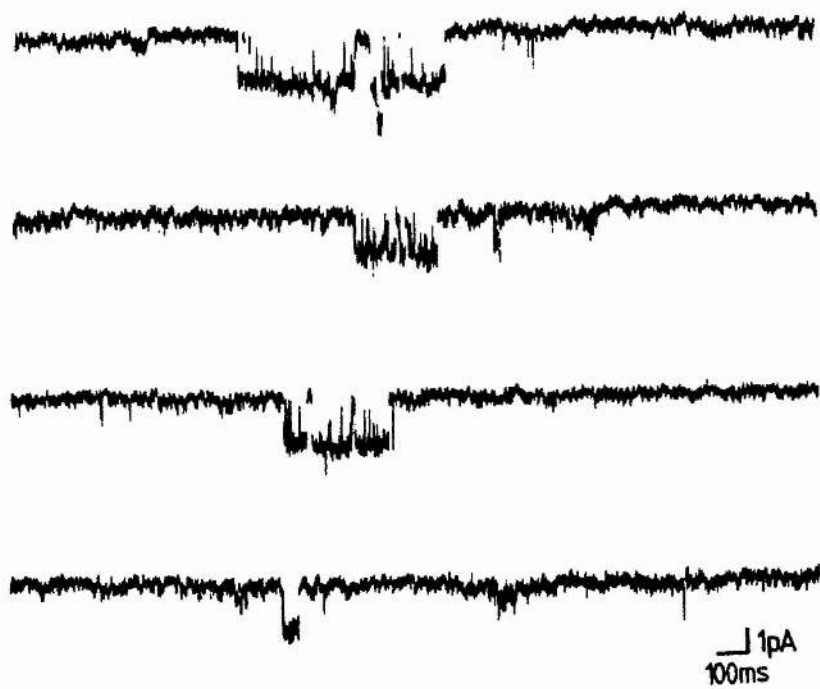
Figure 3.40

Effect of 200nM pregnanolone (5 $\beta$ -pregnane-3 $\alpha$ -ol-20-one) on 1 $\mu$ M GABA-activated unitary currents in an outside-out patch from a mouse spinal neurone.

The 4 traces shown in 1 $\mu$ M GABA represent examples of GABA unitary current activity. The unitary currents represented are the main state of 30pS.

The 4 traces shown in 1 $\mu$ M GABA and 200nM pregnanolone represent typical examples of GABA current activity in the presence of the steroid. The arrows in the last of these traces shows openings to a subconductance level of approximately 21pS. The patch was held at -65mV and the patch pipette contained a solution of 140mM CsCl, while the bath contained a physiological solution of 140mM NaCl.

1 $\mu$ M GABA



1 $\mu$ M GABA and 200nM 5 $\beta$ -PREGNAN-3 $\alpha$ -OL-20-ONE

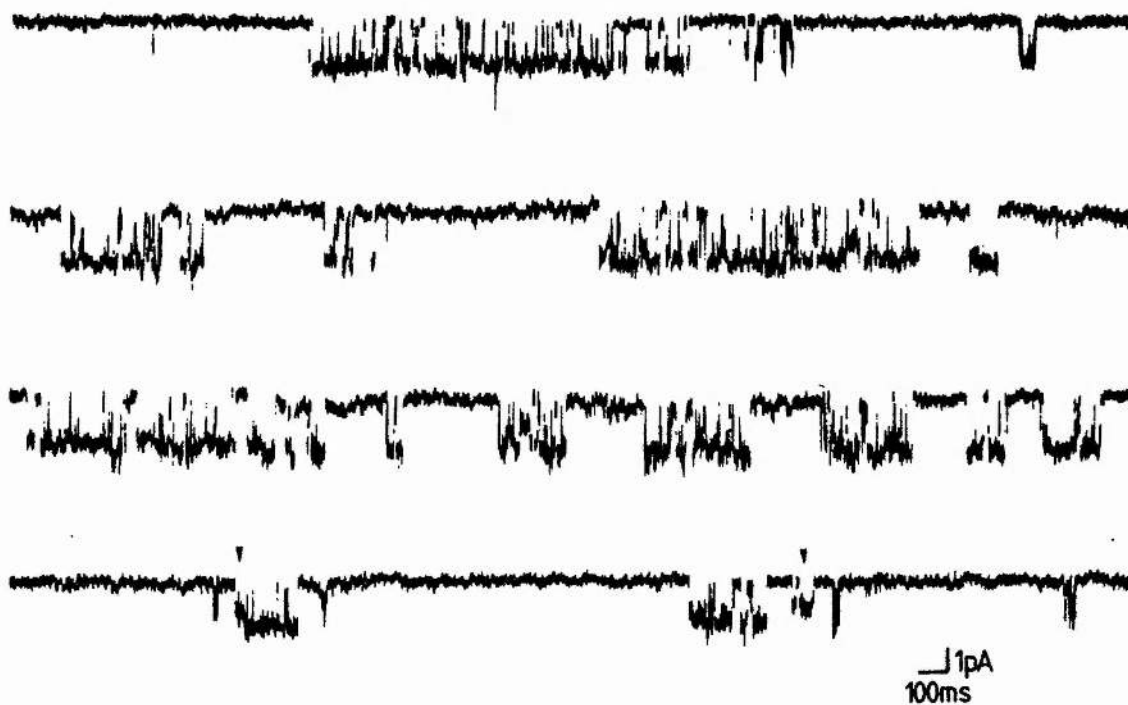




Table 3.1

The mean kinetic parameters obtained from 1 $\mu$ M GABA-activated single-channel currents in the presence and absence of pregnanolone (5 $\beta$ -pregnane-3 $\alpha$ -ol-20-one) 200/300nM. The data were obtained from 3 outside-out patches from mouse spinal neurones. Patch 1 was exposed to 200nM pregnanolone, while the others were exposed to 300nM pregnanolone. The main kinetic parameter affected by the steroid was an increase in the mean burst length.

KINETIC PARAMETER	PATCH 1		PATCH 2		PATCH 3	
	CONT	STER	CONT	STER	CONT	STER
Mean open time (ms)	5.41	8.59	13.69	17.83	3.16	5.65
Mean closed time (ms)	38.35	52.49	12.03	17.73	50.86	23.99
Channel open probability	0.03	0.09	0.11	0.16	0.06	0.22
Mean burst length (ms)	7.98	19.49	16.20	33.70	6.46	48.58
Mean burst length (ms) (excl. single openings)	20.05	32.70	38.60	62.95	23.98	60.08
Mean openings/burst	1.63	2.21	1.36	1.90	1.99	4.77
Mean open time within burst (ms)	6.24	8.77	15.77	18.43	4.85	5.97
Mean open time for single openings (ms)	3.17	7.98	9.69	15.65	1.58	2.88

increases were observed in the other kinetic parameters shown in the table. Most of the channel openings evoked by  $1\mu\text{M}$  GABA were to the 30pS level, openings to sub- and supra-conductance levels were rare. GABA-activated unitary currents in the presence of the steroid often (compared to control) showed openings to the smaller GABA conductance state of 20pS. An example of such openings is shown by the arrows in the bottom trace of the figure.

### 3.11.2 Inability to observe steroid-evoked unitary currents

A weak direct agonist action of the steroids  $5\beta$ pregnane-3 $\alpha$ -ol-20-one and pregnanedione ( $5\beta$ -pregnane-3-20-dione) has been reported on the GABA<sub>A</sub> receptor of chromaffin cells (Callachan et al., 1986). Experiments to determine whether these steroids could also activate the mammalian GABA<sub>A</sub> receptor in outside-out patches from DRG and spinal neurones were attempted. These steroid evoked unitary currents proved difficult to obtain owing to the spontaneous activity described earlier on outside-out patches. Outside-out patches from both types of neurones were exposed to different concentrations of the two steroids by bath perfusion. Table 3.2 shows the number of outside-out patches from DRG and spinal neurones exposed to the different concentrations of the steroids. Upon bath perfusion of the different concentrations of the steroids, no conspicuous difference in the activity of the outside-out patches was noted. Figure 3.41 shows the lack of effect of bath perfusion of  $30\mu\text{M}$  pregnanedione on an outside-out patch from a spinal neurone. The top 2 traces show the activity of the patch in normal physiological solution (control), this patch was then exposed to  $30\mu\text{M}$  pregnanedione by bath perfusion. No

Figure 3.41

The lack of effect of bath perfusion of 30 $\mu$ M pregnanedione (5 $\beta$ -pregnane-3-20-dione) on an outside-out patch from a mouse spinal neurone held at -80mV.

The top two traces show the spontaneous current activity of the patch, the second of which is played out on a faster time scale. The bottom two traces shows the activity of the same patch after perfusing in 30 $\mu$ M pregnanedione, the last trace is again played out on a faster time scale. This patch displayed several conductance states, and the patch pipette contained a solution of 140mM CsCl. The bath contained a physiological solution of 140mM NaCl.

CONTROL



30  $\mu$ M PREGNANEDIONE(5 $\alpha$ -PREGNANE-3, 20-DIONE)



Table 3.2

A summary of the different concentrations of the steroids (pregnanolone and pregnanediolone) exposed to outside-out patches from both mouse spinal and rat DRG neurones. Perfusion of the above concentrations of steroids did not greatly affect the activity of the outside-out patches.

STEROID	<u>NUMBER OF OUTSIDE-OUT PATCHES</u> MOUSE SPINAL NEURONES	RAT DRG NEURONES
<b>PREGNANOLONE</b>		
600nM		2
1.2µM		2
5µM	1	1
10µM	5	
20µM	1	1
100µM		2
<b>PREGNANEDIONE</b>		
30µM	4	

significant difference in the activity of the patch was observed. These results along with the whole-cell studies suggest that these steroids at high concentrations have a weak agonist like action on the mammalian neuronal GABA<sub>A</sub> receptor.

### 3.12 EFFECT OF BETAXALONE(A- $\beta$ -HYDROXY ISOMER OF ALPHAXALONE)

#### 3.12.1 Lack of effect of betaxalone on GABA-evoked whole-cell currents

The 3 $\beta$ -hydroxy isomer of alphaxalone, betaxalone which lacks anaesthetic potency, has been reported to be ineffective in potentiating responses to GABA in the rat cuneate slice preparation (Harrison and Simmonds, 1984). The action of betaxalone on GABA responses of spinal neurones at a concentration of 300nM was investigated; this concentration of alphaxalone reversibly potentiated the GABA current (see figure 3.31).

Betaxalone at 300nM in the bathing solution had no effect on the GABA current in 6 cells. Both betaxalone and alphaxalone at 300nM were exposed to 3 of these cells, alphaxalone reversibly increased the amplitude of the GABA currents whereas betaxalone was ineffective. An example of this effect is shown in figure 3.42.



Figure 3.42

Effect of 300nM betaxalone and 300nM alphaxalone on 30 $\mu$ M GABA-evoked whole-cell currents in a mouse spinal neurone.

The top trace shows a control GABA current in physiological solution. The lack of effect of 300nM betaxalone is represented in the second trace. Alphaxalone at 300nM increased the size of the current (third trace). The effect of alphaxalone was reversed in the wash (bottom trace). The patch pipette contained a solution of 140mM CsCl and the bath contained a physiological solution of 140mM NaCl. The holding potential was -60mV.

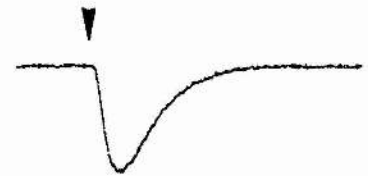
CONTROL



300nM BETAXALONE



300nM ALPHAXALONE



WASH



┐ 1nA  
5s

### 3.12.2 Lack of direct agonist action by betaxalone

Alphaxalone at 50 $\mu$ M in the pressure pipette evoked transient membrane currents in mouse spinal neurones as shown in figure 3.33. A similar effect of betaxalone was studied. Betaxalone, at a concentration of 50 $\mu$ M was pressure applied to spinal neurones held in whole-cell voltage clamp conditions. Betaxalone (50 $\mu$ M) failed to evoke membrane currents in 7 cells, despite using large ejection durations (>100ms). This lack of effect of pressure application of 50 $\mu$ M betaxalone to a spinal neurone held at a range of holding potentials is shown in figure 3.43.

### 3.13 EFFECT OF BEMEGRIDE ON GABA<sub>A</sub> RECEPTOR (WHOLE-CELL STUDY)

Bemegride, a respiratory stimulant was formerly used to counteract barbiturate poisoning in man (Goodman and Gilman, 1965). Some of the actions of the steroids on the GABA<sub>A</sub> receptor (an increase in the burst length of the GABA-activated chloride channels, lack of effect of the benzodiazepine antagonist Ro15-1788) suggested a possible action at or near the barbiturate binding site. To determine whether bemegride could be used as a specific barbiturate antagonist, whole-cell voltage clamp experiments with the barbiturates pentobarbitone and phenobarbitone were performed.

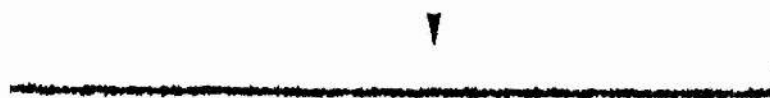
Figure 3.43

The lack of effect of pressure application of 50 $\mu$ M betaxalone on a mouse spinal neurone held in whole-cell voltage clamp conditions. The arrows show where a 350msec pulse of betaxalone was applied to the cell at different holding potentials ( $V_h$ ), no evidence for the activation of a membrane current was obtained. The patch pipette contained a 140mM CsCl solution and the bath contained a physiological solution of 140mM NaCl.

$V_h$  -100mV



$V_h$  0mV



$V_h$  +50mV



└ 50pA  
2s

### 3.13.1 Action of bemegride on phenobarbitone enhanced GABA currents

Experiments were conducted to see whether bemegride could reduce phenobarbitone enhanced GABA-evoked currents in spinal neurones. Figure 3.44 shows the effect of 200 $\mu$ M bemegride on 500 $\mu$ M phenobarbitone increased GABA currents. 200 $\mu$ M bemegride reduced the phenobarbitone enhanced currents. However, the currents in the presence of bemegride took a long time to decay which perhaps indicated an action on the GABA-evoked current rather than any effect on the potentiating action of phenobarbitone. Similar results to that described above were obtained on 5 other cells.

### 3.13.2 Reduction of GABA currents by bemegride

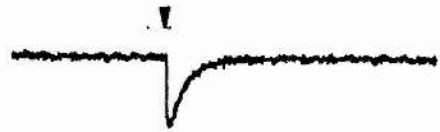
To determine whether bemegride could affect GABA currents on its own, whole-cell experiments with bemegride alone in the bathing solution were performed. Whole-cell inward currents evoked by GABA were reduced upon exposure to 200 $\mu$ M bemegride (figure 3.45). Bemegride reversibly decreased the amplitude by approximately 50% in this cell. In a total of 6 cells studied, bemegride reduced the GABA current by  $54.66 \pm 4.09\%$ . This antagonistic effect of bemegride was not detectably voltage dependent in 3 of these cells outward currents evoked by GABA were reduced by a similar amount. Bemegride and phenobarbitone together was still capable of increasing bemegride reduced currents in 2 cells tested (data not shown), this observation suggested different sites of action for these two drugs.

Figure 3.44

Effect of 200 $\mu$ M bemegride on the phenobarbitone(500 $\mu$ M) potentiated GABA (30 $\mu$ M) current in a mouse spinal neurone.

The top trace shows a control 30 $\mu$ M GABA inward current. In the presence of phenobarbitone the current was enhanced (second trace). With 200 $\mu$ M bemegride and phenobarbitone together in the bathing solution the current was decreased. On washing the inward current was very similar to control. The cell was held at -60mV, the patch pipette contained a 140mM CsCl solution and the bath contained a physiological solution of 140mM NaCl.

CONTROL



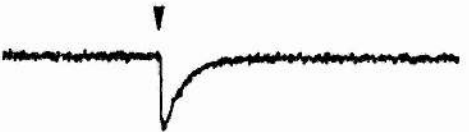
500 $\mu$ M PHENOBARBITONE



500 $\mu$ M PHENOBARBITONE  
+ 200 $\mu$ M BEMEGRIDE



WASH



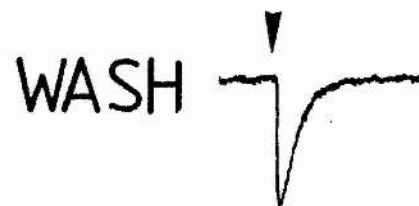
500pA  
5s



Figure 3.45

Effect of 200 $\mu$ M bemegride on the inward current evoked by 30 $\mu$ M GABA in a mouse spinal neurone.

The top trace shows a control inward current evoked by GABA. Perfusion with 200 $\mu$ M bemegride reduced this current by approximately 50% of control (middle trace). This suppression of the current was reversed upon washout (bottom trace). The patch pipette contained a solution of 140mM CsCl and the bath contained a physiological solution of 140mM NaCl. The holding potential was -60mV.



500pA  
10s

### 3.13.3 Pentobarbitone-evoked whole-cell currents

The anaesthetic barbiturate pentobarbitone has been shown to have 2 distinct actions on the mammalian GABA<sub>A</sub> receptor, it can enhance GABA action at low doses and at higher doses it exerts a direct agonist action on the receptor (Akaike, Hattori, Inomata and Oomura, 1985a). Pentobarbitone at high doses (1mM) applied locally by pressure ejection to spinal neurones evoked transient membrane currents. The effect of different holding potentials on the size and sign of the currents evoked by pentobarbitone is shown in figure 3.46. The currents reversed at a holding potential of approximately 0mV. The mean reversal potential was  $-0.3 \pm 1.30\text{mV}$  (n=5). This result suggested that pentobarbitone probably activated a  $\text{Cl}^-$  selective conductance in mouse spinal neurones.

### 3.13.4 Reduction of pentobarbitone currents by bemegride

The action of bemegride on pentobarbitone-evoked whole-cell currents was investigated. Bemegride also reversibly decreased pentobarbitone-evoked whole-cell currents in a manner similar to that found for GABA. An example of the effect of bemegride on pentobarbitone evoked currents is shown in figure 3.47. The mean decrease in the pentobarbitone-evoked current was  $65.2 \pm 5.95\%$  (n=5). No voltage dependence in the reduction was observed outward currents evoked by pentobarbitone were blocked to a similar extent.

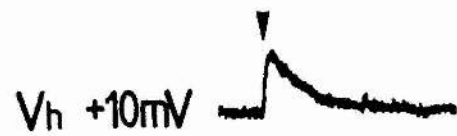
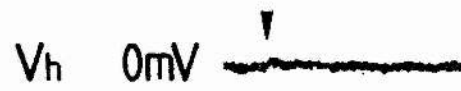
Figure 3.46

Effect of pressure application of 1mM pentobarbitone to a mouse spinal neurone under whole-cell voltage clamp conditions.

The top trace shows an inward current evoked at a holding potential of -20mV. The middle trace shows a small outward current at 0mV and the bottom trace shows a larger outward current at a holding potential of +10mV.

The arrows show where pentobarbitone was applied and the patch pipette contained a solution of 140mM CsCl, while the bath contained a physiological solution of 140mM NaCl.

The relationship between the holding potential and the size and sign of the currents evoked by 1mM pentobarbitone is shown in the graph. Reversal of the currents occurred at approximately 0mV.



┌ 100pA  
└ 2s

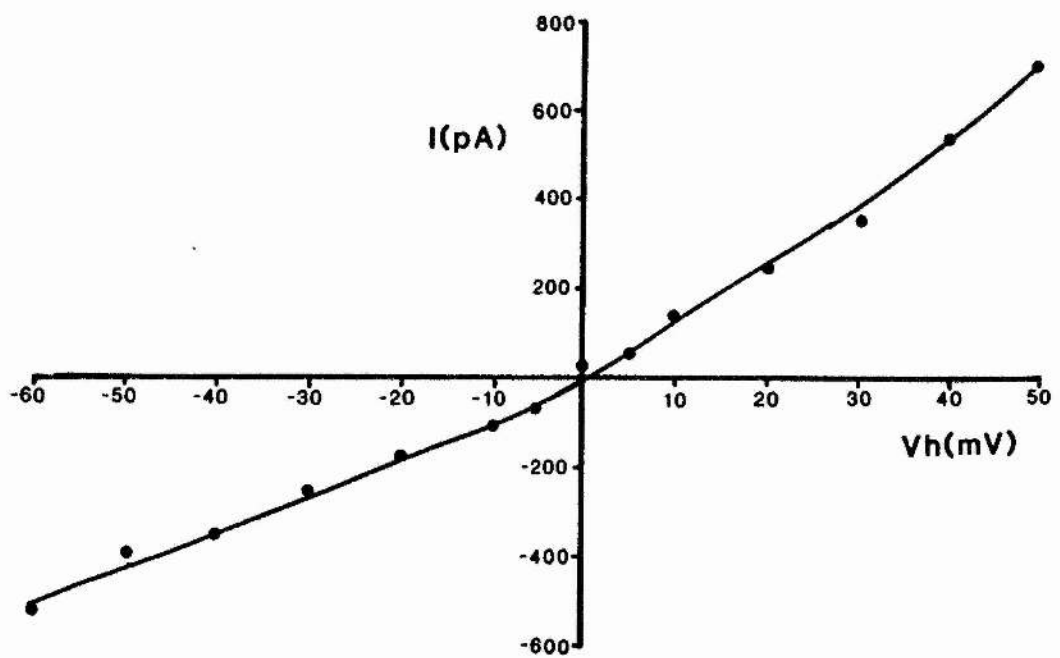


Figure 3.47

Effect of 200 $\mu$ M bemegride on 1mM pentobarbitone-evoked inward currents in a mouse spinal neurone.

The top trace shows a control inward current evoked by pentobarbitone. In the presence of 200 $\mu$ M bemegride this current was reduced in size (middle trace), and this effect of bemegride was reversed upon washout (bottom trace).

The holding potential was -60mV and the patch pipette contained a solution of 140mM CsCl, while the bath contained a physiological solution of 140mM NaCl.

CONTROL 

200 $\mu$ M BEMEGRIDE 

WASH 

 200pA  
2s

### 3.14 CELL-ATTACHED PATCH EXPERIMENTS

Outside-out patches from both mouse spinal and rat DRG neurones often displayed spontaneous  $\text{Cl}^-$  selective single channel currents. Various hypotheses can be formulated to explain their occurrence, some of these include spontaneous openings of the  $\text{Cl}^-$  channels and the loss of intracellular factor(s) upon excision of the patch. Experiments to determine whether spontaneous  $\text{Cl}^-$  selective currents occur in the cell-attached patch mode of mouse spinal and rat DRG neurones were performed.

#### ISOLATION OF $\text{Cl}^-$ CURRENTS.

In spinal neurones the  $\text{Cl}^-$  equilibrium potential is close to the resting membrane potential (Barker and Ransom, 1979). In order to measure a net current through  $\text{Cl}^-$  channels the driving force for  $\text{Cl}^-$  must be increased by reducing the membrane potential across the patch. This results in a net  $\text{Cl}^-$  influx which is measured as an outward current. Spinal neurones upon depolarization, however, activate voltage dependent ion channels. At least three different types of  $\text{K}^+$  channels are turned on at patch potentials more positive than the neurones resting potential. These voltage dependent  $\text{K}^+$  outward currents would interfere with the measurements of  $\text{Cl}^-$  outward currents.

To prevent this interference of  $\text{K}^+$  currents, in cell-attached patches, a solution containing mainly 140mM KCl was used in the patch pipette, this solution was similar to that used by Sakmann, Hamill and Bormann in 1983. When the membrane patch is depolarized by +50 to



+70mV only a small net current flows through the voltage activated  $K^+$  channels, since  $K^+$  is close to its equilibrium potential. Under these conditions  $Cl^-$  currents can be separated from other membrane currents over a limited potential range of  $\pm 20mV$  around 0mV patch potential.

In order to determine the patch potential the resting potential needed to be determined. At the end of the experiment, attempts were made to rupture the membrane under the pipette. The resting membrane potential of the cell was then determined. The patch potential ( $V_p$ ) was calculated by subtracting the potential inside the pipette  $V_{pipette}$  from the membrane potential of the cell ( $V_m$ ). This relationship is shown in equation 1.0

$$V_p = V_m - V_{pipette} \quad 1.0$$

Therefore assuming a membrane potential of -50mV and a pipette potential of -50mV, the potential across the patch ( $V_p$ ) would be 0mV.

### 3.14.1 Cell-attached patches from mouse spinal neurones

17 out of 25 cell-attached patches obtained from different spinal neurones showed unitary current activity, with the patch pipette containing mainly a 140mM KCl solution. Various different types of channel activity and current amplitudes were observed. These unitary current amplitudes reversed in direction at patch potentials between -6 and +20mV, the mean being  $1.4 \pm 4.73mV$  ( $n=9$ ). The unitary currents were inward in direction at patch potentials more negative than the reversal potential, and outward at patch potentials greater than the

reversal potential. No evidence for any outward  $\text{Cl}^-$  currents were found in any of the patches at patch potentials around 0mV.

An example of the type of unitary currents obtained in cell-attached patches and its I-V relationship is shown in figure 3.48. The I-V relationship was linear for a wide range of patch potential (0-90mV) but larger hyperpolarization resulted in a progressive reduction in  $\gamma$  (the single-channel conductance). A similar reduction in the single channel conductance was observed for large calcium-activated potassium channels from cultured rat sympathetic neurones (Smart, 1987).

#### 3.14.2 Cell-attached patches from rat DRG neurones

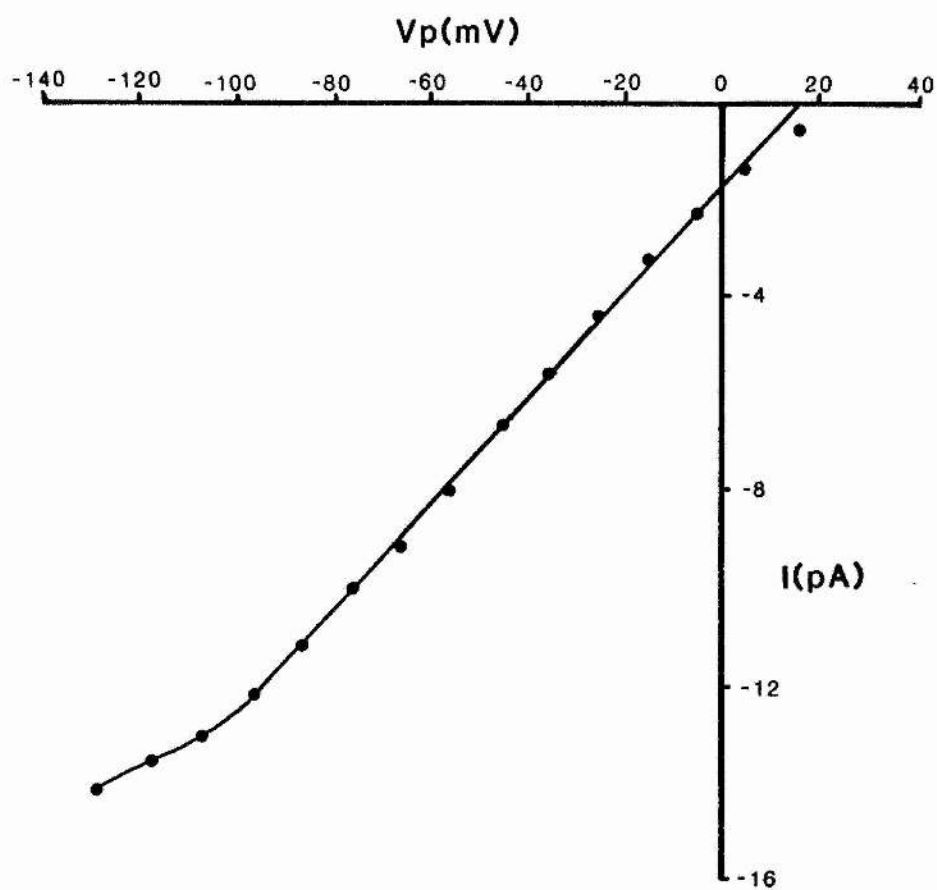
Similar experiments to those described above were performed on rat DRG neurones. A wide variety of channel activity was again observed, which ranged from short open events to opening bursts accompanied by brief and rapid flickerings to the closed state. Some of the channel openings were detectably voltage dependent and several of the cell-attached patches contained more than one type of channel. Simmoneau and co-workers in 1987 have described several different types of  $\text{K}^+$  channels in neonate mouse DRG neurones; these included calcium-activated  $\text{K}^+$  channels, voltage dependent  $\text{K}^+$  channels without  $\text{Ca}^{2+}$  dependence, two types of inward rectifying  $\text{K}^+$  channels and low probability  $\text{K}^+$  channels.

Figure 3.48

Examples of inward currents in a cell-attached patch from a mouse spinal neurone. The patch pipette contained a 140mM KCl solution.

The numbers on the left of each trace represent the patch potential in mV. The resting membrane potential of this cell was -46mV. At a patch potential of -66mV, inward currents of amplitude 9pA were observed these increased to 13pA at -106mV patch potential.

The graph shows the relationship between the patch potential and the unitary current amplitude. The extrapolated reversal potential was +17mV.



Reversal potentials for the unitary currents varied from cell to cell but were in the range -10 to +20mV; the mean being  $10.2 \pm 2.78\text{mV}$  ( $n=20$ ). 29 out of 30 cell-attached patches obtained from different neurones showed channel activity upon depolarization of the patch. In 2 cell-attached patches obtained from mouse DRG neurones with the patch pipette containing 140mM KCl, the mean reversal potential was  $11.0 \pm 4.24\text{mV}$  (mean  $\pm$  S.D.). No evidence for spontaneous  $\text{Cl}^-$  unitary currents were obtained in any of the cell-attached patches. Figure 3.49 shows examples of inward and outward unitary currents in a cell-attached patch from a DRG neurone. The reversal potential of these currents was +17mV, obtained from the I-V relationship. Unitary current amplitudes were linearly related to the patch potential ( $V_p$ ).

#### 3.14.3 Effect of high (300mM) $\text{K}^+$ in the patch pipette

To help identify the nature of the channels observed in the cell-attached patches, the 140mM KCl solution in the patch pipette was replaced with a solution containing mainly 300mM KCl. In 6 cell-attached patches from mouse spinal neurones, the mean reversal potential was  $22.2 \pm 9.07\text{mV}$  in spinal neurones. Assuming a constant internal  $\text{K}^+$  concentration inside the cells and  $\text{K}^+$  selective events; then replacing the 140mM KCl solution with 300mM KCl should shift the  $E_K$  in a positive direction by 20mV. The shift in the mean reversal potential from 1.4mV (140mM KCl) to 22.2mV (300mM KCl) agrees well the suggestion that these channels are  $\text{K}^+$  selective.

Figure 3.49

Examples of inward and outward unitary currents in a cell-attached patch from a rat DRG neurone. The patch pipette contained a 140mM KCl solution and the resting potential was -55mV.

The numbers on the left of each trace represent the patch potential in mV. At a patch potential of -05mV, the unitary currents were inward, while at a patch potential of +45mV the currents were outward. The currents reversed at a patch potential of +17mV, and the I-V relationship was linear.

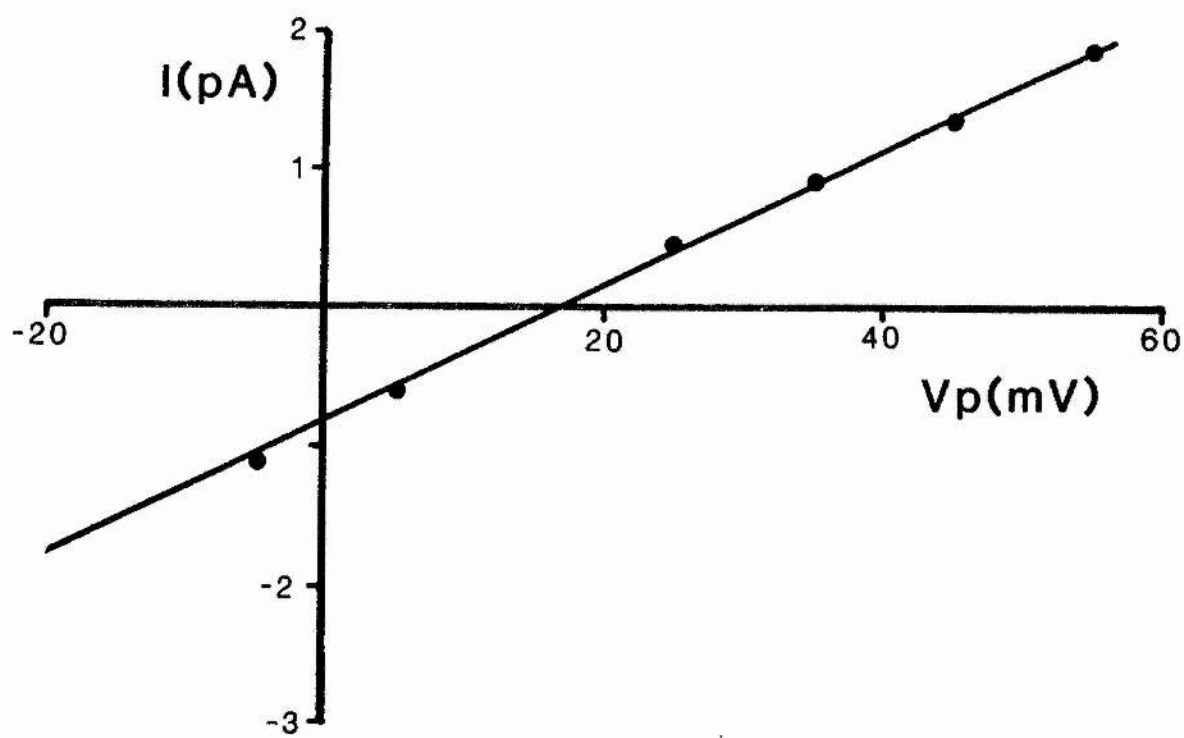


Figure 3.50 shows examples of inward currents obtained in a cell-attached patch with 300mM KCl in the patch pipette. The I-V relationship was linear and had an extrapolated reversal potential of +47mV. Another reason for employing a very high KCl solution in the patch pipette was to increase the size of any possible  $\text{Cl}^-$  currents. The total  $[\text{Cl}^-]$  in this solution was 319mM (see Methods). Bormann, Sakmann and Seifert in 1983 used different  $[\text{Cl}^-]$  in the patch pipette to study GABA-activated chloride channels in rat hippocampal neurones. They reported a linear relationship between the amplitude of the GABA-evoked unitary currents and the  $[\text{Cl}^-]$  within the patch pipette.

#### 3.14.4 GABA-activated unitary currents in cell-attached patches

GABA-activated unitary currents were obtained in cell-attached patches of DRG neurones by adding 2 $\mu$ M GABA to the patch pipette solution (140mM KCl). The patches were again depolarized by 50-60mV inward currents were again found at these potentials, in 4 patches outward currents were also observed. The amplitude of these outward currents increased only slightly with patch depolarization. These outward currents sometimes displayed long openings and different current amplitudes were also observed. Figure 3.51 shows examples of these outward currents and their I-V relationship in a cell-attached patch from a DRG neurone. This patch showed 2 distinct conductance levels. The main state activated by GABA had an amplitude of approx. 0.7pA at a patch potential of 0mV.

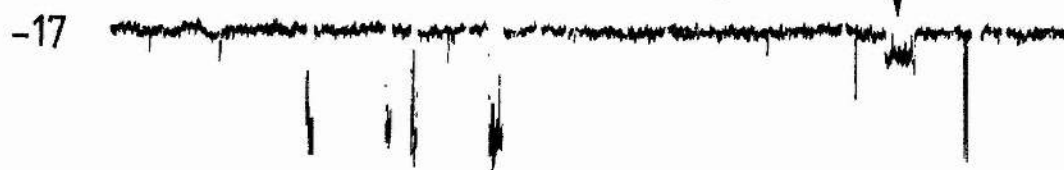


Figure 3.50

Examples of inward currents in a cell-attached patch from a mouse spinal neurone. The patch pipette contained a 300mM KCl solution.

The numbers on the left of each trace represent the patch potential in mV. The resting membrane potential was -47mV. At a patch potential of -17mV, inward currents of approx. 3pA are observed which become smaller in amplitude with patch depolarization. Other smaller current amplitudes were also seen (shown by arrow) on this patch occasionally.

The graph shows the relationship between the patch potential and the unitary current amplitude. Current amplitudes were linearly related to the patch potential and had an extrapolated reversal potential of +47mV



└ 1pA  
100ms

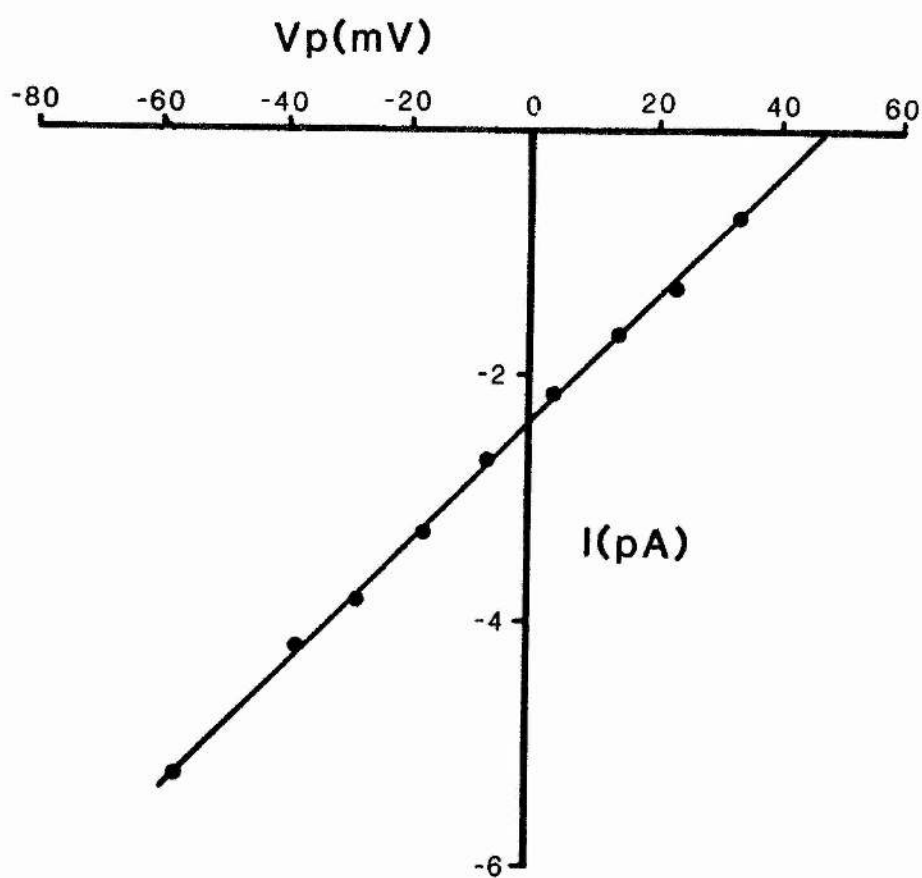


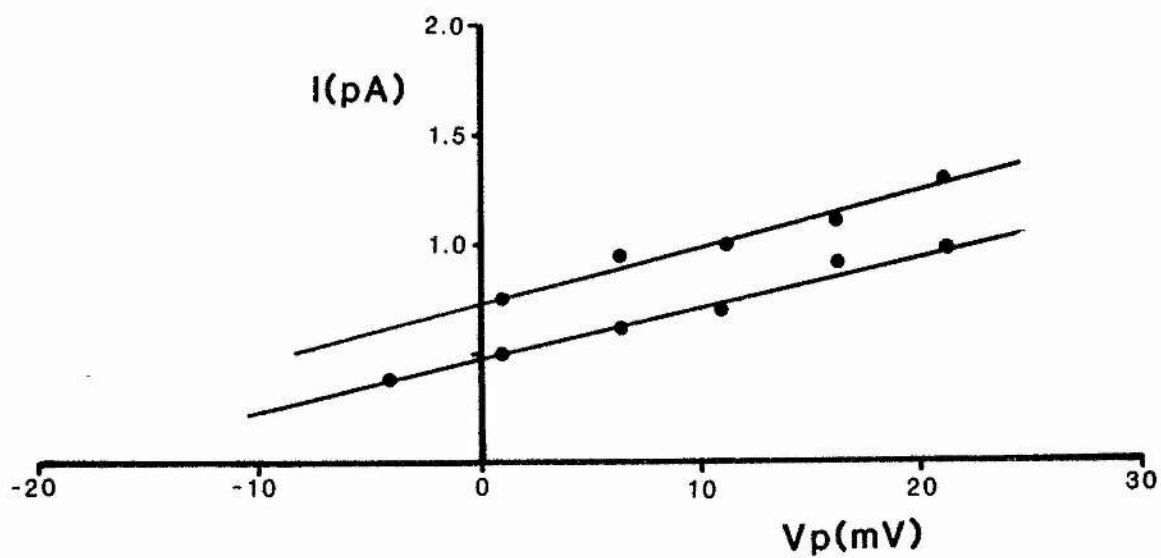
Figure 3.51

GABA-activated outward currents in a cell-attached patch from a rat DRG neurone. The patch pipette contained a 140mM KCl solution with 2 $\mu$ M GABA. The numbers on the left of each trace represent the patch potential in mV and the resting potential was -46mV. On this patch 2 different current levels were observed, the arrow in the bottom trace shows the larger current amplitude of approx. 1.1pA at a patch potential of +16mV.

The graph shows the relationship between the patch potential and the unitary current amplitude. At a patch potential of 0mV GABA-activated unitary currents were of amplitude 0.7 and 0.5pA. No extrapolated reversal potential was obtained since the intracellular Cl<sup>-</sup> concentration was unknown.



┐ 1pA  
100ms



The I-V relationship did not have an extrapolated reversal potential in the graph since the intracellular  $\text{Cl}^-$  activity was unknown but it is thought to be in the range of 30-60mM for peripheral neurones. Central neurones have a lower intracellular  $\text{Cl}^-$  activity, spinal and hippocampal neurones have assumed  $\text{Cl}^-$  activities of 10 and 13mM respectively (Bormann et al., 1983; 1987).



## DISCUSSION

## DISCUSSION

The aim of this thesis was to characterize the GABA<sub>A</sub> response in cultured mammalian neurones. The emphasis was on the pharmacological modulation of the GABA response. The effects of a benzodiazepine, barbiturates and steroids on the GABA response was studied. Single-channel analysis of the effects of the above compounds was hampered by the occurrence of spontaneous unitary events in the outside-out patches. Owing to their frequent occurrence, these spontaneous events were characterized in more detail.

The identity of the cell type could be unambiguously identified using a combination of electrophysiological and morphological criteria (see Methods and Results). Spinal neurones spontaneously fired action potentials, whereas DRG neurones were electrically silent, but they could be made to fire upon injecting depolarizing current.

Almost all of the mouse spinal and rat DRG neurones held in whole-cell voltage clamp conditions responded to pressure application of GABA (10-100 $\mu$ M) with a transient membrane current. The GABA-evoked whole-cell currents in both rat DRG and mouse spinal neurones were reversibly suppressed by bicuculline (2-10 $\mu$ M). Mammalian GABA receptors are divided into two subtypes and are designated GABA<sub>A</sub> and GABA<sub>B</sub> by their selective activation by the GABA agonists, muscimol and baclofen (Bowery, Hill, Doble, Middlemiss, Shaw and Turnball, 1980). GABA<sub>A</sub> receptors are sensitive to bicuculline (Curtis, Duggan, Felix, and Johnston, 1971) and are coupled to Cl<sup>-</sup> channels, while GABA<sub>B</sub> receptors are bicuculline insensitive and are coupled with either Ca<sup>2+</sup> (Dunlap, 1981) or K<sup>+</sup> channels (Gahwiler and Brown, 1985). This



initial pharmacological characterization of the GABA receptor suggested that it was of the GABA<sub>A</sub> type. Bicuculline has been demonstrated to suppress GABA-evoked currents in isolated cone photoreceptors (Kaneko and Tachibana, 1986); in cultured rat melanotrophs (Kehl, Hughes and McBurney, 1987) and in bullfrog DRG neurones (Akaike, Hattori, Oomura and Carpenter, 1985b).

The barbiturate phenobarbitone at 500 $\mu$ M in the bathing solution reversibly potentiated GABA evoked whole-cell currents in spinal neurones. Both the amplitude and the decay time of the currents was increased by phenobarbitone (see figure 3.5). Previous reports have shown that phenobarbitone potentiates GABA action in mouse spinal neurones (Barker and McBurney, 1979, Schultz and MacDonald, 1979). The potentiating action of barbiturates is primarily brought about by a prolongation of the GABA channel open time with little if any effect on the unitary current amplitude as determined from noise analysis techniques (Study and Barker, 1981). Other workers have shown a similar action of phenobarbitone on GABA action in other systems (Cottrell, Lambert and Peters, 1985).

#### 4.1 IONIC NATURE OF THE GABA-EVOKED WHOLE-CELL CURRENTS

The ionic mechanism of the GABA-evoked whole-cell currents was investigated by using both high and low Cl<sup>-</sup> concentrations within the patch pipette. In symmetrically distributed Cl<sup>-</sup> solutions pressure ejection of GABA to spinal neurones evoked outward currents at positive holding potentials and inward currents at negative holding potentials which corresponded to the outward movement of Cl<sup>-</sup>. In all



of the cells obtained, the reversal of the GABA-evoked currents occurred at holding potentials around 0mV. The mean being -3.6mV (n=14). The calculated  $E_{Cl}$  (using  $Cl^-$  activities) was -0.48mV. This reversal potential obtained suggested that GABA-activated a  $Cl^-$  selective conductance. In most of these experiments, the patch pipette contained a 140mM CsCl solution. The  $Cs^+$  was used to suppress unwanted  $K^+$  conductances, however, in a few cases a 140mM TrisCl solution was also used in the patch pipette, but essentially similar results were obtained regarding the reversal potential and the currents evoked by GABA. Experiments identical to those described above were also repeated on rat DRG neurones. Similar results were obtained, outward currents were evoked by GABA at positive potentials and inward currents at negative holding potentials. The mean reversal potential was -0.14mV from 9 cells.

The  $Cl^-$  dependency of the GABA-evoked response was investigated using a low  $Cl^-$  ( $a_{Cl}$  activity 23mM) solution in the patch pipette. The bath still remained a physiological solution containing 140mM NaCl, using these solutions the calculated  $E_{Cl}$  was -38mV. GABA-evoked whole-cell currents were now outward at 0mV, inward at large negative holding potentials and they reversed in direction at holding potentials near -38mV. The mean reversal potential from 9 spinal neurones was -35mV. The above ionic conditions were also used to study the GABA response in rat DRG neurones, here again currents were outward at 0mV and inward at large negative potentials and the currents again reversed around -38mV. In 9 rat DRG neurones, the mean reversal potential was also -35mV. These results provide good evidence that GABA predominantly evoked a  $Cl^-$  selective conductance in these neurones. Other workers have described similar  $Cl^-$  selective

conductance increases to GABA in various cultured cells. For example, in cultured spinal neurones the reversal potential for GABA-activated whole-cell currents shifted by 56mV for a 10 fold change in internal  $\text{Cl}^-$  activity, indicating the activation of  $\text{Cl}^-$  channels. (Bormann, Hamill and Sakmann, 1987). A similar  $\text{Cl}^-$  dependence for GABA-evoked whole-cell currents has also been described in cultured rat melanotrophs (Kehl et al., 1987).

#### 4.2 SPONTANEOUS CURRENTS IN EXCISED PATCHES FROM MOUSE SPINAL NEURONES

Virtually all of the outside-out membrane patches excised from the somata of spinal neurones exhibited spontaneous unitary currents upon isolation. The frequency of these spontaneous events varied from patch to patch, some patches had infrequent events while others had considerable current activity. Within patches, however, there was generally neither a decline of the activity nor periods of increased activity. Sometimes depending on the particular type of experiment being performed, the culture plate was exchanged with physiological solution containing 140mM NaCl between patches. This action, however, did not prevent the occurrence of spontaneous events in the outside-out patches subsequently obtained. In some cases, outside-out patches were deliberately moved well away and across from the cells from which they were obtained, usually they were manoeuvred close to the inlet of the perfusion system. Also some patches were exposed to gentle bath perfusion (1-3ml/minute). In spite of these manipulations no conspicuous difference in the spontaneous unitary current activity was found. The spontaneous current events generally had brief open times but they also occasionally displayed longer lasting opening

events. Spontaneous unitary current events were inward in direction at negative patch potentials ( $V_p$ ) and outward at positive patch potentials with the reversal of these currents occurring at a potential of 0mV. Multiple unitary current levels were observed, but the predominant level varied widely from patch to patch. I-V plots revealed linear I-V relationships for these spontaneous currents. Conductances of these spontaneous events were obtained from the slope of the I-V relationships. In general most of these events had conductance levels smaller than 45pS, but conductances greater than this level were occasionally observed (see figure 3.12). These large conductance states usually displayed brief opening events and had a low frequency of occurrence. The most frequent of the large conductance levels were the 50 and 55pS states, but channels having conductances of 100pS were also identified in a very small number of the patches. These larger conductance levels were also inward at negative patch potentials and outward at positive patch potentials, with reversal occurring at 0mV.

The ionic selectivity of these unitary currents was investigated by using different monovalent cations on one or both sides of the membrane patch. This was done since the spontaneous currents might be attributed to a non-selective cation channel which was equally permeable to  $\text{Cs}^+$  and  $\text{Na}^+$ . Non-selective cation channels have been described in other systems (Yellen, 1982; Maruyama and Petersen, 1982). The impermeant cation Tris was used on one or both sides of the membrane patch. Spontaneous unitary currents were still observed in the outside-out patches with Tris as the main cation. These unitary currents were inward at negative patch potentials and outward at positive patch potentials. Reversal occurred around 0mV, and

multiple conductance states were again found. This result suggested that the spontaneous events were not attributed to non-selective cation channels and that they probably represented the flow of  $\text{Cl}^-$ .

Occasionally, in a few of the patches, clear transitions between different current levels were observed. Some of these transitions involved conductance levels as large as 83pS and as small as 17pS. A general feature of the transitions was that they usually consisted of transitions from a high to a low conductance level rather than vice-versa (see figure 3.16). This particular type of transition is thought to be energetically (thermodynamically) more favourable (Colquhoun- unpublished observations). The occurrence of transitions was not detectably voltage dependent. It is possible that transitions to smaller conductance levels were missed owing to their small size and the difficulty in discriminating a transition from say a rapid closure or a rapid closing and opening of the channel. These clear transitions between different conductance levels provide some evidence to suggest that the spontaneous events might reflect the activity of a  $\text{Cl}^-$  channel which has several substates. Substate activity is usually identified by the following characteristics (for review, see Fox, 1987).

- (1) A channel substate should interconvert with the channel mainstate, thus direct transitions from one conductance level to another should be observed.
- (2) The substate should only be seen in the presence of channel mainstate activity.
- (3) One must exclude the possibility that the main state is the superposition of two independent channels, for example, to determine whether 100pS channel has a 60pS substate, 60pS and 100pS transitions,

but NO transitions to 40pS must be observed.

The first and second statements are somewhat difficult to address in this case, since the mainstate varied from patch to patch. However, overall the 21pS and 30pS conductance levels were the most frequently occurring and transitions involving these states to other sub- and supra-conductance levels were seen (see figure 3.16).

The spontaneous unitary currents observed in the outside-out patches were presumably  $\text{Cl}^-$  selective. Experiments with the low  $\text{Cl}^-$  solution in the patch pipette helped to support this suggestion. The bath remained a physiological solution containing 140mM NaCl, using these solutions the calculated  $E_{\text{Cl}}$  was -38mV. The mean reversal potential of the spontaneous unitary currents shifted from 0mV to a value close (-36mV) to the calculated  $E_{\text{Cl}}$ . The I-V relationships of the spontaneous currents were non-linear and was well fit by the theoretical Goldmann Hodgkin and Katz (GHK) equation for a  $\text{Cl}^-$  current, using permeability values of  $7.7 \times 10^{-14}$  and  $10.7 \times 10^{-14} \text{ cm}^3 \text{ s}^{-1}$  and intracellular and extracellular  $\text{Cl}^-$  activities of 23 and 105mM respectively. Unitary currents were outward at positive patch potentials and inward at large negative patch potentials, multiple current levels were again found.

The nature of these spontaneous events was investigated with bicuculline. Bath perfusion with bicuculline (10 $\mu$ M) blocked spontaneous current activity in 5 outside-out patches. This finding suggested that most if not all of the spontaneous events occurred from the operation of GABA-sensitive ion channels.

#### 4.3 SPONTANEOUS CURRENTS IN EXCISED PATCHES FROM RAT DRG NEURONES

As with the spinal neurones, isolated outside-out membrane patches from DRG neurones also displayed spontaneous unitary currents upon excision from the cell. Considerable similarities were found between the properties of these spontaneous events and those already described in outside-out patches from spinal neurones. Firstly, the frequency of the spontaneous currents varied widely from patch to patch. In symmetrically distributed  $\text{Cl}^-$  solutions, the I-V relationship was linear with currents being inward at negative patch potentials and outward at positive patch potentials. Reversal of these spontaneous currents occurred at 0mV, suggesting that they were  $\text{Cl}^-$  selective. As with spinal neurones, multiple current levels were found which had conductances ranging from 6 - 67pS.

Using different monovalent cations on one or both sides of the membrane patch did not change the reversal potential or the current amplitudes of the spontaneous events. Various different monovalent cations were used which included  $\text{Na}^+$ ,  $\text{Tris}^+$ ,  $\text{K}^+$  and  $\text{Choline}^+$ . The experiments with a low  $\text{Cl}^-$  solution in the patch pipette further confirmed the  $\text{Cl}^-$  selectivity of these events. The reversal potential was shifted to a value close to the calculated  $E_{\text{Cl}}$ . The I-V relationship was non-linear and was again well fit by the theoretical GHK equation for a  $\text{Cl}^-$  current using permeability values and intracellular and extracellular  $\text{Cl}^-$  activities. Multiple conductance states were again found.



Distinct transitions between various different conductance levels were also occasionally observed. Owing to the short open times, of the spontaneous currents, careful analysis of the patches had to be performed in order to spot these transitions. The occurrence of the transitions did not appear to be voltage dependent. Most of the transitions involved the main 30pS state. Transitions from this state to other conductance levels as low as 13pS and as high as 56pS were found (see figure 3.23). Transitions to smaller conductance levels were probably missed because of their small size and the difficulty in determining between a sojourn at these levels and a rapid closing and opening of the channel.

The nature of the spontaneous unitary currents was investigated with bicuculline, as in the case for spinal neurones, bicuculline (10 $\mu$ M) blocked the spontaneous activity in the outside-out patches. This finding coupled with the Cl<sup>-</sup> selectivity of these events strongly suggest that these currents reflect the activity of GABA-sensitive channels.

#### 4.4 CELL-ATTACHED PATCH EXPERIMENTS

An insight into the possible nature of the spontaneous events was investigated using cell-attached patches from spinal and DRG neurones. In order to observe any possible Cl<sup>-</sup> currents, a high K<sup>+</sup> (140mM KCL) solution was employed in the patch pipette and the patch had to be depolarized to  $E_K$  approximately 0mV. Sometimes after forming the cell-attached patch at resting potential, unitary inward

currents were found, and this current activity often increased with patch depolarization. Patch depolarization often also activated other different types of inward currents. These unitary currents reversed in direction at patch potentials near 0mV ( $E_{rev}$ ), at potentials more positive than  $E_{rev}$  these currents were outward. I-V relationships of these currents were linear but the reversal potential varied somewhat from cell to cell. The reversal potential near 0mV suggested that these currents probably carried  $K^+$ , further evidence to support this was obtained using a 300mM KCl solution in the patch pipette. The mean reversal potential of these unitary currents shifted by +21mV. This shift was very close to the predicted shift (20mV) in the  $E_K$  as calculated from the Nernst equation assuming  $K^+$  selective events and a constant intracellular  $K^+$  concentration. This result strongly suggested that the unitary currents found in the cell-attached patches represent the activity of different types of  $K^+$  channels. The variability in the reversal potential of the  $K^+$  currents is probably due to the variation in the intracellular  $K^+$  concentration from cell to cell. A variation in the intracellular  $K^+$  concentration in different frog motoneurons was shown by Burhle and Sonnhof in 1983 using  $K^+$  sensitive microelectrodes.

Different types of  $K^+$  channels have been demonstrated in neonate mouse DRG neurones (Simmoneau, Distasi, Tauc and Barbin, 1987), these included calcium activated  $K^+$  channels, voltage dependent  $K^+$  channels, two types of inward rectifying  $K^+$  channels and low probability  $K^+$  channels. Cultured mouse spinal neurones (Aguayo and Albuquerque, 1987) have also been shown to have different types of  $K^+$  channels.



In both the mouse spinal and rat DRG neurones no evidence for outward currents representing the possible flow of  $\text{Cl}^-$ , was found at potentials near the  $E_K$  in the absence of GABA in the patch pipette. This was the case when the patch pipette contained either 140mM KCl or 300mM KCl solutions. Bormann et al. (1987) using a similar high  $\text{K}^+$  solution in the patch pipette also reported a lack of  $\text{Cl}^-$  currents in the absence of GABA and glycine in the patch pipette. The findings from the cell-attached patches suggest that the spontaneous  $\text{Cl}^-$  currents found in the excised outside-out patches do not reflect spontaneous openings of  $\text{Cl}^-$  channels and that the spontaneous  $\text{Cl}^-$  channels observed in the excised patches probably do not reflect the activity of voltage dependent  $\text{Cl}^-$  channels.

Spontaneous openings of the acetylcholine (ACh) receptor channel, however, have been described in cell-attached patches from embryonic rat muscle cells (Jackson, 1984). Treatment of the cells with  $\alpha$ -bungarotoxin blocked these spontaneous currents. A reactive disulfide bond near the receptor binding site was reduced with dithiothreitol and alkylated with N-ethylmaleimide. This action did not reduce the frequency of the spontaneous openings but did inhibit suberyldicholine and curare activated channel currents. He concluded that the ACh receptor briefly and infrequently fluctuated into an active state in the absence of agonist, and that the presence of agonist accelerates this spontaneous process.

Outside-out patches obtained from mouse spinal neurones and rat DRG neurones with the patch pipette containing a 140mM KCl solution, displayed outward currents at patch potentials more positive than 0mV. Some of these outward currents were detectably voltage dependent. In some patches, the outward currents were as large as 5pA in amplitude at a patch potential of 0mV. In one patch, these large amplitude outward currents were reversibly decreased in size by the  $K^+$  channel blocker tetraethylammonium at 10mM. These observations in outside-out patches suggest that different types of  $K^+$  channels are present in spinal and DRG neurones.

#### 4.5 GABA-ACTIVATED UNITARY CURRENTS IN MOUSE SPINAL NEURONES

Pressure application of GABA (10-100 $\mu$ M) to the outside-out patches displaying spontaneous activity evoked unitary currents in all of the patches tested (n=9). The number of GABA-activated channels present on a patch varied widely, some had just one or two while others had anything up to 10 channels present. At negative patch potentials application of GABA evoked inward unitary currents in symmetrical  $Cl^-$  solutions. The main state activated by GABA was one around 30pS, but other smaller and larger conductance levels were also seen occasionally. Bath perfusion of low concentrations of GABA (1-10 $\mu$ M) enabled a more direct study of these multiple conductance states. Generally, bath perfusion of GABA (1-10 $\mu$ M) to outside-out patches displaying low spontaneous activity resulted in the activation of only a few (1-2) channels. Bath perfusion of GABA to outside-out patches displaying frequent spontaneous activity, evoked considerable

channel activity with the activation of at least three channels and multiple openings were often found. Exposure of outside-out patches which displayed low spontaneous activity, to GABA resulted in an increased frequency of opening events and the appearance of bursting activity. The most frequent conductance state activated by GABA was the 30pS level, but in the patch shown in figure 3.20 distinct openings to conductance levels of 20 and 44pS were also found, with the former being more frequent. Low concentrations of GABA were also exposed to outside-out patches isolated in asymmetrical  $\text{Cl}^-$  solutions, here also GABA increased the frequency of opening events and caused bursting activity. GABA-evoked unitary currents of approximate amplitude 1.3 and 2.2pA at a patch potential of +47mV, these currents were outward in direction. Pressure application of GABA (10-100 $\mu\text{M}$ ) to outside-out patches also evoked transient outward currents again some variability in the number of GABA channels present on the patches was observed (see figure 3.19).

#### 4.6 GABA-ACTIVATED UNITARY CURRENTS IN RAT DRG NEURONES

Outside-out membrane patches from rat DRG neurones responded to pressure application of GABA (10-50 $\mu\text{M}$ ) with the activation of unitary currents. All of the patches showing spontaneous current activity responded to the application of GABA. As with outside-out patches from spinal neurones, there was considerable variability in the number of channels present on a patch. Pressure application of GABA to outside-out patches activated unitary currents whose amplitude was linearly related to the patch potential (see figure 3.26). The slope of the I-V relation revealed that the main state activated by GABA was

around 30pS and the extrapolated reversal potential was 0mV indicating  $\text{Cl}^-$  selective events. Apart from this main conductance level, other smaller levels of around 13 and 20pS were also activated by GABA.

Multiple conductance states were also found in cell-attached patches from DRG neurones with 2 $\mu\text{M}$  GABA in the patch pipette. The outward currents evoked by GABA could only be seen in a narrow range of potentials owing to the contaminating  $\text{K}^+$  currents which were also outward in direction at positive potentials (see Results). An extrapolated reversal potential could not be ascribed to the GABA-activated currents, since the intracellular  $\text{Cl}^-$  concentration was unknown. It is generally thought that the  $\text{Cl}^-$  concentration in DRG neurones is higher than that found in central neurones (approx. 13mM) (Bormann et al., 1983). The results suggest that the  $\text{GABA}_A$  receptor of rat DRG neurones is capable of adopting multiple conductance levels in both cell-attached and outside-out patch modes. Multiple conductance levels of the  $\text{GABA}_A$  and glycine receptors have also been described in both of these recording modes in cultured spinal neurones (Bormann et al., 1987).

#### 4.7 SPONTANEOUS UNITARY CURRENTS OBSERVED BY OTHER WORKERS.

Several other workers have reported spontaneous events in excised outside-out patches from mouse spinal neurones (Hamill, Bormann and Sakmann, 1983; Mathers, 1985, 1987; Weddle and MacDonald, 1985; Rogers, Twyman and MacDonald, 1987; Barker, Dufy, Harrison, Owen and MacDonald, 1987a). Strychnine (100-500nM) was employed in the bathing solution to block glycine coupled  $\text{Cl}^-$  events, in spite of this measure

spontaneous events were still found in outside-out patches (Rogers et al., 1987). Weddell and Macdonald in 1985 had previously shown that these events were  $\text{Cl}^-$  selective by replacing some of the  $\text{Cl}^-$  in the bath solution with the impermeant anion isethionate, the reversal potential of these events shifted to the  $E_{\text{Cl}}$ . This suggested that  $\text{Cl}^-$  was the principal charge carrier. Picrotoxin also blocked these spontaneous events. Barker et al. (1987a) also described spontaneous unitary  $\text{Cl}^-$  currents. Conductance levels of 30 and 50pS were found and clear transitions between these two states were observed.

Spontaneous unitary currents have also been found in excised patches in other systems (Weiss, Barnes and Hablitz, 1988; Taleb, Trouslard, Demeneix, Feltz, Bossu, Dupont and Feltz, 1987). Infrequent spontaneous events have been described in isolated outside-out patches from cultured chick cerebral neurones (Weiss et al., 1988). These neurones were shown to be insensitive to excitatory amino acids at up to 2 weeks in culture and also to be insensitive to glycine. Perfusion of outside-out patches with 1 $\mu\text{M}$  GABA increased the activity of the patches and caused bursting activity to appear. Spontaneous unitary currents in both the whole-cell and the outside-out patch mode were described in cultured pituitary intermediate lobe cells from the pig (Taleb et al., 1987). These workers using symmetrically distributed  $\text{Cl}^-$  solutions, showed that these spontaneous events reversed around 0mV and that they had a linear I-V relationship between -100mV and +40mV. Anything up to 7 distinct current levels were observed in the outside-out patches having approximate conductances of 6, 11, 15, 24, 29, 38, and 44pS. The most frequently occurring states were the 11 and the 26pS levels. These spontaneous unitary currents had brief open times and the

level varied from patch to patch. These events were shown to be  $\text{Cl}^-$  selective by replacing some of the internal  $\text{Cl}^-$  with the impermeant anion gluconate or  $\text{SO}_4^{2-}$ , the spontaneous events now reversed at the calculated  $E_{\text{Cl}}$ . No effect of intracellular monovalent cations was observed on the amplitude of these unitary currents.

#### 4.8 SPONTANEOUS LARGE CONDUCTANCE LEVELS

Unitary currents having conductances greater than 45pS have been shown in isolated outside-out patches from spinal neurones (Barker et al., 1987a; Hamill et al., 1983). The nature of the large conductance events described in this thesis are unknown. Recently a report by Hughes, McBurney, Smith and Zorec (1987) described multiple conductance states of 21, 30, 45, 66, and 92pS activated by external  $\text{Cs}^+$  (50-100mM) in cultured rat spinal neurones. The unitary currents activated by  $\text{Cs}^+$  were  $\text{Cl}^-$  selective. The 3 smallest conductance levels activated by  $\text{Cs}^+$  were very similar to the conductances activated by both GABA and glycine (McBurney, Smith and Zorec, 1985). Since large conductance levels had been previously demonstrated in mouse spinal neurones (Hamill et al., 1983). These authors suggested that  $\text{Cs}^+$  was activating the same type of channel as both glycine and GABA. This question was addressed by Smith (1987) using the whole-cell voltage clamp technique. He found that bicuculline had little or no effect on the  $\text{Cs}^+$  activated  $\text{Cl}^-$  current, but strychnine reduced this current by 70-85%. It was proposed that  $\text{Cs}^+$  activated the same type of  $\text{Cl}^-$  channel as the neurotransmitter glycine.



It is possible that some or all of the spontaneous events described in this study could be attributed to glycine coupled  $\text{Cl}^-$  currents. The experiments whereby the GABA antagonist bicuculline blocked the spontaneous activity argues against this notion. Other workers have also described a similar action of bicuculline on the spontaneous  $\text{Cl}^-$  currents in outside-out patches from spinal neurones (Mathers, 1987; Barker, Dufy, Harrison, Owen and MacDonald, 1987a). The latter workers showed that bicuculline blocked the 30 and 50pS states similarly. These findings suggest that the spontaneous events seen in the outside-out patches primarily reflect the activity of GABA sensitive  $\text{Cl}^-$  channels. However, it is still possible that the spontaneous events are glycine coupled  $\text{Cl}^-$  currents since glycine responses in mouse spinal neurones have been shown to be sensitive to bicuculline (Martin, McHanwell, and Biscoe, 1978). Bicuculline was also capable of blocking the spontaneous current activity in outside-out patches from pituitary intermediate lobe cells (Taleb et al., 1987).

In this study, the distinct transitions between the different conductance levels suggest that most if not all of the spontaneous conductance levels are part of a large macromolecular complex having several substates and do not reflect the activity of different types of channels. Also, all the different conductance levels show similar kinetics in terms of the open and closed times, corroborating the notion that they might represent substates of a large molecular complex. Figures 3.12 and 3.16 show transitions between small conductance levels (<45pS) and large conductance levels of 82pS, 67pS and 73pS in outside-out patches from mouse spinal neurones. A

considerable proportion of the transitions involved the 3 most frequent states of 20, 30 and 45pS. Transitions between different conductance levels in outside-out patches from rat DRG neurones were also found (figure 3.23). Here again transitions between large conductance and smaller conductance levels were observed, for example, the second trace in the figure shows a transition between conductance levels of 56 and 31pS. Most of the transitions involved the most frequent conductance levels of 20 and 30pS.

#### 4.9 SIMILARITIES BETWEEN SPONTANEOUS EVENTS AND GABA-ACTIVATED CURRENTS

Comparing the conductances of the GABA-activated unitary currents with those of the spontaneous events, a striking similarity was found. Firstly, the most frequent spontaneous conductance states in outside-out patches from both spinal and DRG neurones were the 30 and 20pS levels. The main unitary currents activated by GABA in both spinal and DRG neurones also had conductance values of around 20 and 30pS.

It has now been shown that at least 4 conductance states of 12, 19, 30 and 45pS exist for the GABA<sub>A</sub> receptor in mouse spinal neurones (Bormann et al., 1987). In this study as well as the above states, intermediate conductance levels of 7 and 37pS were also found as well as larger levels greater than 45pS in the outside-out patches from spinal neurones.



In outside-out patches from DRG neurones as well as spontaneous events with conductances similar to that activated by GABA (13, 20, and 30pS), other conductance states of around 7, 36, 42, 51, 59 and 67pS were also found. Rat DRG neurones have been shown to possess both GABA<sub>A</sub> and GABA<sub>B</sub> receptors (Desarmenien, Desaulles, Feltz and Hamann, 1987), but are not thought to be sensitive to excitatory amino acids or glycine. Taleb et al. (1987) also described some similarities between the spontaneous conductance states and those activated by GABA, 3 of the spontaneous conductance states were very similar to those activated by GABA. In other studies, conductance states of 6-8pS (Weiss et al., 1988) and 37pS (Cottrell et al., 1985) have been found for the GABA<sub>A</sub> receptor. In recent years, more and more conductance states have been described for the GABA<sub>A</sub> receptor in cultured cells, the main difference between the various cells appears to be the mainstate activated by GABA. The similarities between the conductances activated by GABA and the spontaneous events in both spinal and DRG neurones suggest that the spontaneous events reflect the activity of GABA-sensitive chloride channels. The study of GABA-activated unitary currents in outside-out patches from rat DRG neurones was somewhat limited because it was difficult to keep these patches for more than a few minutes. It could be possible that the GABA<sub>A</sub> receptor of these cells is also capable of activating conductance levels greater than the mainstate of 30pS.

#### 4.10 POSSIBLE EXPLANATIONS FOR THE SPONTANEOUS EVENTS.

The spontaneous events described in this thesis have been shown to be  $\text{Cl}^-$  selective, but the exact identity of these currents is open to debate. Some if not all of the spontaneous events could possibly be attributed to a totally different type of  $\text{Cl}^-$  channel from say the GABA-gated one. It might perhaps be a voltage dependent  $\text{Cl}^-$  channel or a calcium dependent  $\text{Cl}^-$  channel. A  $\text{Ca}^{2+}$ -dependent chloride conductance has been reported in cultured spinal neurones (Owen, Segal, and Barker, 1984, 1986) and in cultured rat DRG neurones (Mayer, 1985).

The spontaneous  $\text{Cl}^-$  currents described in this thesis are probably not  $\text{Ca}^{2+}$  dependent since analysis of the tail current kinetics show that the channel open times are as long as 100ms plus these currents were only demonstrated by using  $[\text{Ca}^{2+}]_i$  in the  $\mu\text{M}$  range (Mayer, 1985). Also it seems unlikely that the spontaneous unitary currents represent the activity of a  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  channel since  $\text{Ca}^{2+}$  was deliberately omitted from most of the patch pipette solutions and the  $\text{Ca}^{2+}$  chelating agent EGTA was included in all of the pipette solutions. The only pipette solution to have any appreciable  $[\text{Ca}^{2+}]$  was the low  $\text{Cl}^-$  solution. The free  $\text{Ca}^{2+}$  concentration of this solution was calculated using a microcomputer program which determined the free  $\text{Ca}^{2+}$  concentration of solutions (Nicol, 1985). Using this program a value of  $3.28 \times 10^{-8}$  M was calculated. Initial experiments with outside-out patches from spinal neurones were also performed using an intracellular physiological solution identical to that used by Fenwick, Marty and Neher, (1982). A free  $[\text{Ca}^{2+}]$  of  $3.35 \times 10^{-8}$  M was calculated for this 140mM KCl solution. No conspicuous difference

was observed in the activity of these patches compared to those obtained with a negligible intracellular  $[Ca^{2+}]$ .

Intracellular  $Ca^{2+}$  has been shown to affect GABA-activated  $Cl^-$  channels in frog sensory neurones (Akaike, Ikemoto, Koneda and Ono, 1987). In these neurones, an increase in the intracellular  $[Ca^{2+}]$  concentration decreased the probability of opening of GABA-activated chloride channels. An interaction of intracellular  $Ca^{2+}$  has also been shown with the spontaneous  $Cl^-$  channels in pituitary intermediate lobe cells (Taleb et al., 1987). Outside-out patches obtained from these cells showed only infrequent spontaneous activity with a  $[Ca^{2+}]_i$  of  $10^{-9}M$ . In contrast to this, with  $[Ca^{2+}]_i$  of  $10^{-8}$  or  $10^{-7}M$ , a much higher rate of activity was found, however no spontaneous activity was found with  $[Ca^{2+}]_i$  of  $10^{-6}M$ . Similar results to that described above were found for GABA-activated unitary currents in these cells, with  $[Ca^{2+}]_i$  of  $10^{-6}M$  isoguvacine (GABA agonist) evoked little activity, but with  $10^{-8}$  or  $10^{-7}M$   $[Ca^{2+}]_i$  GABA and isoguvacine evoked much greater activity. These results suggest that intracellular free  $Ca^{2+}$  can exert a direct control on the opening of  $Cl^-$  channels whether they are spontaneously open or are activated by GABA. It remains to be seen whether this control is also present in other mammalian systems.

Apart from the possible role of intracellular  $Ca^{2+}$ , the loss of other intracellular factors may also possibly play a role in accounting for the spontaneous currents. The  $\beta$ -subunit of the  $GABA_A$  receptor has recently been shown to possess a cAMP dependent serine phosphorylation consensus sequence (Scholfield et al., 1987). A cAMP dependent kinase has been reported to specifically phosphorylate the  $\gamma$  and  $\delta$  subunits of the acetylcholine receptor. In this receptor

phosphorylation increases the probability of channel opening without affecting the single-channel conductance (Montal, 1987). The increase in the channel opening was brought about by a decrease in the long closed time that separates bursts of channel opening. It is not known what effect phosphorylation has on the channel kinetics of the GABA<sub>A</sub> receptor, excision of the patch could perhaps lead to a loss of phosphorylation of the receptor which may increase the probability of channel opening. Another possible explanation of the spontaneous events is that excision of the membrane patch might somehow affect the receptor protein complex causing it to spontaneously open. Although the spontaneous Cl<sup>-</sup> currents are probably not Ca<sup>2+</sup> dependent, it cannot be discounted that they are not some sort of voltage dependent Cl<sup>-</sup> channel. A voltage dependent Cl<sup>-</sup> conductance has been described in mouse spinal neurones (Owen et al., 1986), but the relative distribution of the channels comprising this conductance in excised patches remains unknown. The data emerging from the experiments with the isolated outside-out patches from both spinal and DRG neurones suggest that these spontaneous Cl<sup>-</sup> currents probably reflect the activity of GABA sensitive ion-channels.

There appears to be close similarities between the glycine and the GABA receptor, and there have been suggestions that they might operate the same ion channel. Barker and McBurney (1979) showed that macroscopic conductance responses to GABA and glycine in mouse spinal neurones displayed competition. Considerable similarities in the conductances activated by these inhibitory transmitters have now been reported with single-channel recording techniques (Bormann et al., 1987; McBurney, Smith and Zorec, 1985). Four similar conductance levels have been described for these two transmitters, but each

transmitter activates its own preferential conductance level. Similar anion permeabilities have also been shown for these two transmitters (Bormann et al., 1987).

This is similar to the finding for excitatory amino acids in central neurones. (Cull-Candy and Usowicz, 1987; Jahr and Stevens, 1987). These workers showed at least 5 conductance states for these amino acids. Glutamate and aspartate preferentially activated levels of 40-50pS. NMDA predominantly activated conductance levels above 30pS, while quisqualate and kainate mainly activated ones below 20pS. The presence of clear transitions between the different levels suggested that the 5 main levels were all substates of the same channel. There now appears to be a great deal of homology between the ligand gated ion channels (Stevens, 1987) in terms of the amino acids making up the different receptors. The similarities between the conductances activated by GABA and glycine in spinal neurones, (McBurney et al., 1985; Bormann et al., 1987), suggest great structural similarities between the ion-channels of these two receptors. If  $\text{Cs}^+$  is indeed acting directly on the glycine receptor (Smith, 1987) then this receptor apart from activating the more usual conductance levels (20, 30, 45pS) can also somehow gate larger conductance levels of 66 and 92pS. This notion if correct suggests that the glycine receptor is a larger macromolecular complex than at first thought. It might even be plausible that the  $\text{GABA}_A$  receptor is also somehow capable of adopting large conductance levels greater than 45pS. In one isolated outside-out patch from a mouse spinal neurone large conductance levels of 97, 90, 67 and 55pS were seen in the presence of 1 $\mu\text{M}$  GABA /1 $\mu\text{M}$  GABA and 200nM pregnanolone. Smith (1987a) described a large conductance level of 71pS for the GABA channel in

rat spinal neurones. This finding supports the suggestion that the GABA receptor might very occasionally be capable of adopting large conductance levels.

#### 4.11 EFFECT OF DIAZEPAM ON GABA-EVOKED WHOLE-CELL CURRENTS

The benzodiazepine agonist diazepam enhanced GABA-evoked whole-cell currents in spinal neurones. Three different concentrations of diazepam were used 10nM, 1 $\mu$ M and 10 $\mu$ M. 10nM diazepam enhanced GABA currents in only 2 out of the 11 cells tested. It is thought that the serum levels of diazepam are generally in the low  $\mu$ M range, diazepam is highly (94-99%) bound to the plasma proteins and the free plasma and cerebrospinal fluid concentrations of diazepam are thought to be in the 5-120nM range (Eadie and Tyrer, 1980; Kanto, Kangas and Sirotola, 1975). Enhancement of GABA responses by diazepam in this concentration range have been demonstrated in frog sensory neurones (Hattori, Oomura, and Akaike, 1986).

A possible reason for the low number of cells responding to 10nM diazepam may lie in the expression of different benzodiazepine receptors in the spinal cord. As well as peripheral type benzodiazepine binding sites in the adult rat spinal cord (Villiger, 1985), there is also some evidence for heterogeneity of the central benzodiazepine receptor (Villiger, 1984). Both type 1 and type 2 benzodiazepine receptors were found in the spinal cord using Cl 218872 binding but the exact proportions of these two receptors is unknown. Recent evidence suggests that these two types of central receptors represent different conformations of the same macromolecular complex



(Sato and Neale, 1987). If so, then it might be possible that the response to diazepam depends on the particular configuration of the benzodiazepine receptor. For example, one conformation would favour an inverse agonist while another would prefer an agonist (Braestrup, Nielsen, Honore, Jensen and Petersen, 1983). If the former state was the one most frequently encountered it might explain the lack of effect of agonists.

Higher concentrations of diazepam (1 and 10 $\mu$ M) produced more consistent responses, but here again some 11% of the cells showed no response to diazepam. There was very little difference between the increase in the size of the GABA currents caused by these two concentrations. Other workers have also described a lack of benzodiazepine action on the GABA<sub>A</sub> receptor in the  $\mu$ M range (Vicini, Alho, Costa, Mienville, Santi and Vaccarino, 1986; Biscoe and Duchon, 1986). This lack of action of benzodiazepines on the benzodiazepine receptor remains a puzzle, but the possibility cannot be excluded that some intracellular regulating factor such as receptor turnover could be involved.

Only relatively small increases in the size of the GABA current were demonstrated, with little or no effect on the decay time of the currents. This finding is consistent with the view that the primary action of diazepam was to increase the frequency of channel opening (Study and Barker, 1981). Application of GABA to the cell in the presence of diazepam would increase the probability that the channel was open, thereby creating a larger initial current, however, once open since diazepam has little action on the channel opentime there would be little effect on the decay time of the whole cell currents.

#### 4.12 EFFECT OF STEROIDS ON THE GABA<sub>A</sub> RECEPTOR

Alphaxalone at low doses in the bathing solution reversibly increased the amplitude and the decay time of GABA-evoked whole-cell currents in both mouse spinal and rat DRG neurones. In other studies, this enhancement of GABA action has been shown to be insensitive to Ro15-1788 suggesting that it does not act at the benzodiazepine receptor site (Cottrell, Lambert and Peters, 1986; Harrison and Simmonds, 1984). The increase in the amplitude of the GABA currents in spinal neurones was dose dependent, but with concentrations of alphaxalone greater than 1 $\mu$ M, an increase in the baseline current noise was also observed indicating a direct action on the membrane. This direct action was subsequently investigated using pressure ejection, it was found that alphaxalone (10-50 $\mu$ M) alone was capable of evoking a whole-cell membrane Cl<sup>-</sup> current. These Cl<sup>-</sup> currents were affected by drugs known to interact directly (bicuculline) and allosterically (phenobarbitone) with the GABA<sub>A</sub> receptor. It is concluded that alphaxalone in the  $\mu$ M range somehow is capable of directly activating the GABA<sub>A</sub> receptor.

Possible reasons for the relatively low increase in the size of the GABA currents in rat DRG neurones caused by 600nM alphaxalone include: (a) the GABA<sub>A</sub> receptor might be slightly different from the central GABA<sub>A</sub> receptor (b) The alphaxalone used in these experiments was over 1 year old and may have lost some of its activity. The potentiation of GABA action by low doses of alphaxalone has been demonstrated in several mammalian preparations. Alphaxalone at concentrations greater than 30nM in the bathing medium dose-dependently increased GABA-evoked whole-cell currents in the



bovine chromaffin cell (Cottrell, Lambert and Peters, 1987b). Harrison and Simmonds (1984) also described potentiation of GABA action by alphaxalone in the rat cuneate slice preparation. Similar observations have also been reported in rodent central neurones (Barker, Harrison, Lange and Owen, 1987b; Cottrell, Lambert and Mistry, 1987a). The direct agonist like action of alphaxalone at high doses has been shown in recordings from bovine chromaffin cells (Cottrell et al., 1987b). Noise analysis techniques applied to cultured rat spinal neurones, revealed that GABA and alphaxalone activated similar  $\text{Cl}^-$  channels with an approximate conductance of 20pS (Barker et al, 1987b). Unitary currents evoked by 10 $\mu\text{M}$  alphaxalone in outside-out patches from bovine chromaffin cells had a similar conductance (30pS) to those activated by GABA (Cottrell, Lambert and Peters, 1987b).

Alphaxalone was originally synthesized from observations that certain endogenous steroids and their metabolites were very potent anaesthetics. Some of these steroids included progesterone and its metabolites 5 $\beta$ -pregnane-3 $\alpha$ -20-dione (pregnanedione) and 5 $\beta$ -pregnane-3 $\alpha$ -ol-20-one (pregnanolone). Pregnanolone proved to be very potent at enhancing GABA-evoked whole-cell currents in spinal neurones. Both the amplitude and the decay time of the GABA currents were markedly increased, with concentrations as low as 30nM in the bathing medium. The increase in the size of the GABA currents was dose-dependent with the mean enhancement being 887% in the presence of 100nM pregnanolone. As with alphaxalone, a direct agonist like action of pregnanolone was investigated. Pressure application of 10 $\mu\text{M}$  pregnanolone onto spinal neurones evoked small long-lasting currents, in spite of using long pulse durations and high pressures. These

currents reversed in direction at 0mV possibly indicating the involvement of a  $\text{Cl}^-$  conductance.

Various other workers have also shown an interaction of endogenous steroids with the  $\text{GABA}_A$  receptor. Both pregnanolone (10-300nM) and pregnanediolone (300nM-10 $\mu$ M) have been reported to reversibly potentiate GABA currents in bovine chromaffin cells (Callachan, Cottrell, Hather, Lambert, Nooney and Peters, 1986). The increase in the GABA currents was not affected by Ro15-1788. At higher steroid concentrations pregnanolone (1-30 $\mu$ M) and pregnanediolone (1-30 $\mu$ M) directly evoked small whole-cell currents in these cells. These currents were reversibly suppressed by bicuculline and potentiated by phenobarbitone. Endogenous steroids have also been shown to affect the  $\text{GABA}_A$  receptor in mammalian neurones. Pregnanolone, 5 $\alpha$ -pregnane 3 $\alpha$ -ol-20-one (3 $\alpha$ -OH DHP), and 5 $\beta$ -pregnane 3 $\alpha$ -21-diol-20-one (THDOC) pressure ejected onto rat spinal and hippocampal neurones increased GABA activated  $\text{Cl}^-$  currents and ipsos (Harrison, Majewska, Harrington and Barker, 1987a).

#### 4.13 MECHANISM OF POTENTIATION OF GABA RESPONSES BY STEROIDS

The mechanism by which GABA-evoked currents were potentiated by steroids was investigated at the single-channel level using outside-out patches from spinal neurones. There was no detectable difference in the amplitude of GABA-activated single-channel currents in the presence of 200/300nM pregnanolone. GABA-activated unitary currents, however, tended to open in long bursts in the presence of pregnanolone. Kinetic analysis of the unitary currents in the

presence and absence of pregnanolone showed that the main parameter affected was the mean burst length. Other kinetic parameters were also affected but to a lesser extent. The mean open time of single openings was increased slightly in the presence of the steroid. The mean open probability was also slightly increased, but this value may be a considerable overestimate. The large increase in the burst length contributes significantly to the total open time and hence the probability of opening  $P(0)$ .

$$P(0) = \frac{\text{total open time}}{\text{total open time} + \text{total closed time}}$$

In fact, careful inspection of the outside-out patches suggest that the frequency of channel opening is decreased if anything in the presence of steroid. However, when the channel did open it often displayed burst like behaviour as shown in figure 3.40. An interesting finding in the presence of pregnanolone was that openings of the channel to the 20pS subconductance level were more frequent. This state was entered from the channel closed state as well as from the channel main state (30pS), openings to this conductance level is shown in the bottom trace of figure 3.40. The only other report available on the single-channel mechanism of potentiation by steroids on GABA action was performed using outside-out patches from bovine chromaffin cells (Callachan, Cottrell, Hather, Lambert, Nooney and Peters, 1987a). Using a similar concentration of pregnanolone (300nM) which in whole-cell studies would markedly potentiate GABA currents. These authors showed that the main effect of the steroid was also to greatly prolong the burst length of the GABA-activated unitary currents. The probability of the channel opening was increased nearly 3 fold, and openings to the 20pS level also appeared to be more

frequent when compared to the control.

The action of pregnanolone and presumably other steroids on GABA-activated unitary currents is similar to that seen with barbiturates. Barbiturates, as well as increasing the burst length and individual channel openings, also increased the frequency of channel openings in outside-out patches from mouse spinal neurones (Twyman, Rogers and MacDonald, 1987). Some of the macroscopic actions of the steroids appear to correlate well with the single-channel studies. The primary action of alphaxalone on GABA-evoked ipscs in cultured rat hippocampal neurones (Harrison, Vicini and Barker, 1987b) was to increase the decay time of these currents. There was a small decrease if anything in the peak amplitude of the ipscs. This increase in the decay time also extends to the metabolites 3-OH-DHP and THDOC (Harrison et al., 1987a). These actions of the steroids are clearly different from that of the benzodiazepine agonists, for example, flunitrazepam prolonged the decay and increased the amplitude of GABA-evoked ipscs in cultured rat cortical neurones (Vicini et al., 1986). The results suggest that steroids have little if any effect on the frequency of channel opening caused by GABA. The main action of the steroid appears to be the stabilization of the GABA-activated ion channel in the open conformation.

Alphaxalone (1 $\mu$ M) prolonged the mean open time of GABA-activated ion channels in cultured rat spinal and hippocampal neurones as determined from noise analysis techniques (Barker, Harrison, Lange, Majewska and Owen, 1986). The prolongation of GABA channel burst duration by steroids most likely accounts for the potentiation of macroscopic GABA-evoked responses caused by steroids found in

mammalian cells

#### 4.14 WEAK DIRECT AGONIST ACTION OF ENDOGENOUS STEROIDS

Application of 10 $\mu$ M pregnanolone to spinal neurones evoked only a small membrane Cl<sup>-</sup> current, attempts at characterizing unitary currents evoked by endogenous steroids proved to be unsuccessful. Bath perfusion of pregnanolone (600nM-100 $\mu$ M) and pregnanedione (30 $\mu$ M) to isolated outside-out patches from DRG and spinal neurones were attempted. However, no convincing evidence for any steroid-evoked unitary currents were obtained from any of the patches. A few of the patches had appreciable spontaneous activity and it is possible that this activity prevented the identification of steroid-evoked unitary currents. The whole-cell studies coupled with the single-channel studies suggest that these steroids have a weak agonist like action on the GABA<sub>A</sub> receptor. Unitary currents evoked by 30 $\mu$ M pregnanedione, however, have been obtained in outside-out patches from chromaffin cells (Callachan et al., 1987a). These unitary currents had a similar conductance and reversal potential to those activated by GABA and these currents were also antagonized by bicuculline. It cannot be excluded that the GABA<sub>A</sub> receptor of these cells are somewhat more susceptible to steroids, since in vivo these cells are surrounded by the adrenal cortex which releases various different steroids including progesterone, corticosterone and aldosterone into the bloodstream. The exact physiological significance of the presence of GABA<sub>A</sub> receptors in bovine chromaffin cells is unknown. The relatively weak agonist action of pregnanolone and pregnanedione might possibly be attributed to the slight difference in the structure of these

molecules compared to alphaxalone. The =O bond at C-11 in alphaxalone might be important in conferring agonist like properties on the GABA<sub>A</sub> receptor.

#### 4.15 SITE OF ACTION OF STEROIDS

Barbiturates and steroids in radioligand binding studies have very similar actions on the GABA<sub>A</sub> receptor. They both inhibit TBPS binding (Ramanjaneyula and Ticku, 1984; Harrison et al., 1987a), they also stimulate the binding of the benzodiazepine flunitrazepam and GABA and its analogues (Olsen and Snowman, 1982; Majewska, Bisslerbe and Eskay, 1985). Several electrophysiological aspects of steroid action on the GABA<sub>A</sub> receptor resemble that of pentobarbitone. This barbiturate anaesthetic enhances GABA action at low concentrations but at higher doses (300µM-3mM) it also is capable of activating the GABA<sub>A</sub> receptor (Akaike, Hattori, Inomata, and Oomura, 1985a; Barker and Owen, 1986). Both, steroids and pentobarbitone increase GABA action by prolonging GABA channel opentime (Segal and Barker, 1984; Gage and Robertson, 1985; Harrison, Vicini and Barker, 1987b). Pentobarbitone evoked Cl<sup>-</sup> currents can be modulated by drugs which interact directly (bicuculline) and allosterically (picrotoxin and diazepam) with the GABA<sub>A</sub> receptor (Akaike, Maruyama, and Takutomi, 1987; Barker and Owen, 1986).

The interaction of bicuculline is interesting since bicuculline is thought to be a competitive antagonist of the mammalian GABA<sub>A</sub> receptor (Simmonds, 1983). The finding that the direct agonist action of these drugs can be blocked by bicuculline perhaps suggest that the



direct agonist action occurs at or near the GABA recognition site. Moreover, it is unlikely that they act at the same site since 1mM pentobarbitone-evoked whole-cell currents can be markedly increased by 500nM pregnanolone and the reciprocal interaction also occurs. Unitary currents in excised outside-out patches can be prolonged by 500 $\mu$ M phenobarbitone in bovine chromaffin cells (Callachan, Lambert and Peters, 1987b). Presumably, 1mM pentobarbitone would saturate the pentobarbitone binding site, if steroids also acted at this site then they would compete with pentobarbitone. Since the steroids are still capable of potentiating pentobarbitone-evoked whole-cell currents it is unlikely that they occupy a common site of action; the reciprocal interaction also occurs occupation of the steroid site does not prevent barbiturate action.

A general perturbation of the lipid bilayer caused by the steroids dissolved in the membrane could possibly account for their action on the GABA<sub>A</sub> receptor. The experiments with betaxalone the  $\beta$ -hydroxy isomer of alphaxalone which lacks anaesthetic activity showed that this compound failed to potentiate GABA-evoked whole-cell currents in spinal neurones. Betaxalone also lacked an agonist like action on the GABA<sub>A</sub> receptor. Several other reports have been made on the lack of effect of betaxalone on the GABA<sub>A</sub> receptor, for example, in the rat cuneate slice preparation (Harrison and Simmonds, 1984); in the bovine chromaffin cell (Cottrell et al., 1987b); in cultured rat hippocampal neurones (Harrison, Vicini and Barker, 1987b) and in cultured rat spinal neurones (Barker et al., 1987b). The endogenous pregnane steroids also show a similar structural selectivity in that the 3 $\beta$ -hydroxy analogues are ineffective at potentiating GABA action, while the 3 $\alpha$ -hydroxy analogues are very active in potentiating GABA

responses (Kirkness, Lambert, Peters and Turner, 1987; Harrison et al, 1987a). This strict stereoselectivity argues against a general disordering of the lipid bilayer caused by the steroids dissolved in the membrane. The present information available suggests that the action of the steroids on the GABA<sub>A</sub> receptor occurs at a distinct receptor site which is different from the benzodiazepine and the barbiturate binding sites.

#### 4.16 ACTION OF BEMEGRIDE ON THE GABA<sub>A</sub> RECEPTOR

The main purpose for the experiments with bemegride, a respiratory stimulant, was to see whether it could be used as a barbiturate antagonist. Bemegride (200 $\mu$ M) was capable of reversibly reducing the phenobarbitone enhanced GABA current. Phenobarbitone has been shown to prolong the decay time of the GABA-evoked currents. The GABA-evoked currents in the presence of bemegride, still had a rather prolonged decay time, which was similar to that observed with phenobarbitone alone. This finding suggested that bemegride had an action on the GABA-evoked current rather than any preferential effect on the phenobarbitone action. This possible action of bemegride was addressed by looking at its effect on GABA currents alone. GABA-evoked whole-cell currents were reversibly suppressed by 200 $\mu$ M bemegride. This antagonistic action of bemegride on the whole-cell currents evoked by GABA was not detectably voltage dependent. GABA-evoked outward currents were blocked to a similar extent as the inward currents.



Pentobarbitone (1mM), directly evoked a membrane  $\text{Cl}^-$  current in mouse spinal neurones as shown in figure 3.46. Bemegride at a concentration of 200 $\mu\text{M}$ , reversibly reduced the size of the pentobarbitone-evoked current. The % reduction in the size of the current was very similar to that observed for the GABA-evoked  $\text{Cl}^-$  currents, again no voltage dependence in this reduction was found. Bemegride appeared to have a non-competitive antagonist action on the GABA<sub>A</sub> receptor. The finding that GABA and pentobarbitone evoked currents were similarly reduced by bemegride suggests that this compound acts at a separate site from the barbiturate one. The clinical actions of bemegride may have been brought about by a "convulsant like" action on the GABA<sub>A</sub> receptor. This compound does not appear to act as a specific barbiturate antagonist and hence it was not used to elucidate site of steroid action on the GABA<sub>A</sub> receptor.

In overview, the potentiating effects of diazepam and phenobarbitone on GABA responses have confirmed the findings of other workers. In the last couple of years steroids have been shown to have two distinct actions on the GABA<sub>A</sub> receptor. At low concentrations they potentiate GABA action and at higher doses somehow directly activates the GABA<sub>A</sub> receptor. The mechanism by which steroids potentiate GABA action was studied at the single-channel level. The main action of the steroids was to prolong the burst length of the GABA-activated unitary currents in outside-out patches. This action on the unitary currents was clearly different from that caused by benzodiazepine agonists, but similar to that observed with barbiturates. Bemegride, a respiratory stimulant was used clinically

to counteract barbiturate poisoning. Experiments were made to determine whether bemegride could be used as a barbiturate antagonist. These experiments, however, showed that bemegride appeared to have a non-competitive action on the GABA<sub>A</sub> receptor.

Outside-out membrane patches from both spinal and DRG neurones revealed spontaneous current activity. These spontaneous unitary currents had various different conductances and were shown to involve the flow of Cl<sup>-</sup>. Other workers have recorded spontaneous events in outside-out patches where the GABA<sub>A</sub> receptor is present (Barker et al., 1987a; Taleb et al., 1987; Weiss et al., 1988) and in the whole-cell configuration (Taleb et al., 1987). However, experiments with the cell-attached patches showed that spontaneous Cl<sup>-</sup> currents did not occur. These findings suggest that dialysis of the cell with the contents of the patch pipette may lead to a loss of an intracellular factor(s) causing spontaneous events. A recent review has described an important role for protein phosphorylation in receptor function (Huganir and Greengard, 1987). Phosphorylation has been shown to increase the probability of opening of the nicotinic acetylcholine receptor (Montal, 1987). Since other neurotransmitters can regulate the activity of various protein kinases this may be a way of regulating the activity of the acetylcholine receptor. It should be noted that the spontaneous events may not be physiologically important because:

(a) of their lack of occurrence in the intact cell and (b) the neurones are in the relatively strange domain of tissue culture.

However, if an intracellular factor(s) does indeed exert a control on the activity of the GABA<sub>A</sub> receptor, then the identity of this factor(s) would be potentially very interesting in terms of modulation of the receptor through the cytoplasmic surface.



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# APPENDIX

Membrane permeability to non-electrolytes is usually defined by the empirical flux equation:

$$M_s = -P_s \Delta C_s$$

Flux equals permeability times the concentration difference. If  $s$  is the permeant molecule;  $M_s$  (mole.cm<sup>-2</sup>s<sup>-1</sup>) is the molar flux density of  $s$ ;  $P_s$  is the membrane permeability to  $s$ ;  $\Delta C_s$  (mole.litre<sup>-1</sup>) is the concentration gradient.

Rearranging the equation gives units of cm.s<sup>-1</sup> for  $P_s$ .

In the Goldman Hodgkin and Katz (GHK) equation, because the particles (ions) are charged, the flux is determined both by the concentration gradient and by the electric field (Hodgkin and Katz, 1949). The GHK equation is based on the assumptions that the potential drop across the membrane is linear and that ions move independently of each other.

In the single-channel experiments, the absolute current through the channels was measured and the permeability calculated. Since the current calculated was not expressed in terms of the unit area (cm<sup>-2</sup>) of membrane, the units of permeability are therefore cm<sup>3</sup>s<sup>-1</sup>. The permeability of the channel was used, since the conductance depends on the ionic conditions and the patch potential (especially in asymmetrical solutions). The permeability values were calculated using a microcomputer program from the equation:

$$P_{Cl} = \frac{i_{Cl} RT(1 - e^{-\frac{VF}{RT}})}{F^2 V (Cl_i - Cl_o) e^{-\frac{VF}{RT}}}$$

where  $P_{Cl}$  is the permeability value (cm<sup>3</sup>s<sup>-1</sup>);  $i_{Cl}$  is the single



channel current (pA);  $F$  is the Faraday constant ( $\text{CM}^{-1}$ ) (96500);  $R$  is the gas constant ( $\text{J}^{\circ}\text{K}^{-1}.\text{mol}^{-1}$ ) (8.31);  $T$  is the absolute temperature ( $^{\circ}\text{K}$ ); and  $V$  is the patch potential (mV);  $\text{Cl}_o$  and  $\text{Cl}_i$  are the concentration (activities) of Cl ions outside and inside the membrane respectively.